Faculty of Engineering of University of Porto

Integrated Master in Bioengineering

Microencapsulation of Chlorogenic acid – a natural antioxidant

Dissertation for Master degree in Biological Engineering

Bruna Daniela Barrias Gonçalves

Supervisor: Dr. Lúcia Santos
Co-Supervisor: Dr. Berta Estevinho

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To my Parents and Brother
To my Godmother
To João

“Intelligence is the ability to adapt to change.”
Stephen Hawking

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Abstract

Coffee is one of the most consumed and commercialized food products in the world which, in a chemical point of view, has gained an increased interest due to its biologically active compounds. Among them, polyphenols, in particular chlorogenic acids (CGA) are highlighted because of their beneficial health effects. Besides their important role in the quality, flavor and bitterness of coffee, CGAs carry relevant nutritional and functional properties mainly associated with their antioxidant activity. Moreover, CGAs present a large number of interesting biological activities such as anti-inflammatory, antimicrobial, antiviral, among others. Their contribution in the prevention of several diseases associated with oxidative stress, such as cancer, premature aging, strokes, Alzheimer's, Parkinson's, and other cardiovascular and neurodegenerative diseases have been also documented in the literature. However, due to their structural and chemical nature, CGAs can easily undergo oxidation. Furthermore, they are instable under high temperature conditions and they may suffer from transesterification reaction, limiting their industrial applications. For these reasons, the stabilization of CGA is one of the major concerns. Therefore, CGAs requires a finished formulation able to maintain their structural integrity until its consumption, mask its taste, increase its bioavailability and control its release after administration. Among the existing stabilization methods, microencapsulation has been widely reported as an efficient solution. There are several techniques of microencapsulation but, due to its low cost, availability of equipment and efficiency, spray drying is one of the most used encapsulation technique in food industry. Biopolymers, such as sodium alginate and modified chitosan, are encapsulating agents with an increased interest for food applications.

The aim of the present study was to prepare and characterize sodium alginate and modified chitosan microparticles of chlorogenic acid in order to evaluate their controlled release at two different pH values (pH 5.6 and 2) and their enhanced bioavailability. The microparticles were prepared by spray drying and characterized by their particle size and surface morphology by particle size distribution through laser granulometry (Coulter) and SEM analysis, respectively. High performance liquid chromatography (HPLC-DAD) and UV-Vis spectrophotometry methods were developed and validated for analytical detection and quantification of chlorogenic acid. Both methods presented good linearity with correlation coefficients (R^2) better than 0.999 and good precision results with coefficients of variation values lower than 5%. The limits of detection (LOD) and quantification (LOQ) for HPLC were 0.19 $mg.L^{-1}$ and 0.64 $mg.L^{-1}$, respectively. For UV-Vis spectrophotometry, the limits of detection and quantification were 0.18 $mg.L^{-1}$ (LOD) and 0.60 $mg.L^{-1}$ (LOQ) in water at pH 5.6, and 0.09 $mg.L^{-1}$ and 0.31 $mg.L^{-1}$ in water at pH 2, respectively. Through this work we conclude that it is possible to encapsulate chlorogenic acid with sodium alginate and modified chitosan as encapsulating agents using

spray drying technique. Satisfactory product yields of 41.1% and 39.3% were obtained for production of sodium alginate and modified chitosan microparticles, respectively. Particle size distribution in volume (%) revealed a mean size of 2.8 µm and 3.2 µm for sodium alginate and modified chitosan microparticles. Their shape presented to be spherical with a smooth surface. Controlled release studies of chlorogenic acid (3-CQA) showed a fast release for both types of microparticles in water at both pH values. Results obtained by HPLC and UV-Vis spectrophotometry were concordant about the release of chlorogenic acid trough sodium alginate microparticles. For modified chitosan, 80% of 3-CQA was released in 10 min, approximately. The release rate of chlorogenic acid (3-CQA) was not significantly affected by pH. As a first study, results are significant and reported the success of chlorogenic acid microencapsulation for food-matrices application. However more studies should be done in order to improve even more the controlled release pattern of chlorogenic acid in food products.

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Keywords: Coffee, Chlorogenic acid, Microencapsulation, Spray drying, Sodium alginate, Modified chitosan, Controlled release, Food industry

Resumo

O café é um dos produtos alimentares mais consumidos e comercializados em todo o mundo que, de um ponto de vista químico, ganhou um interesse acrescido devido aos seus compostos biologicamente activos. Entre eles, os polifenóis, em particular os ácidos clorogénicos (ACGs), são destacados pelos seus efeitos benéficos para a saúde. Para além do seu papel importante na qualidade, sabor e amargura do café, os ACGs possuem propriedades nutricionais e funcionais relevantes, associadas principalmente à sua actividade antioxidante. Além disso, ACGs apresentam um grande número de actividade biológicas interessantes tais como actividade anti-inflamatória, antimicrobiana, antivírica, entre outras. A sua contribuição na prevenção de várias doenças associadas com o estresse oxidativo, tais como cancro, envelhecimento prematuro, acidentes vasculares, Alzheimer, Parkinson e outras doenças cardiovasculares e neurodegenerativas têm também vindo a ser documentada na literatura. Contudo, devido à sua natureza estrutural e química, ACGs sofrem facilmente oxidação. Além disso, são instáveis em condições de elevadas temperaturas e podem sofrer reacção de transesterificação, limitando as suas aplicações industriais. Por estas razões, é importante garantir a estabilidade dos ACGs numa formulação final capaz de manter a sua integridade estrutural até ao seu consumo, mascarar o seu sabor, aumentar a sua biodisponibilidade a controlar a sua libertação após a sua administração. Entre os possíveis métodos de estabilização existentes, a microencapsulação tem sido amplamente divulgada como uma solução eficiente. Existem várias técnicas de microencapsulação, mas o *spray drying* é uma das técnicas de encapsulação mais utilizadas na indústria alimentar, devido ao seu baixo custo de processo, disponibilidade de equipamento e eficiência. Biopolímeros, como o alginato de sódio e quitosano modificado, são exemplos de agentes encapsulantes com interesse acrescido em aplicações alimentares.

O objectivo do presente estudo foi preparar e caracterizar micropartículas de ácido clorogénico encapsulado por alginato de sódio e quitosano modificado de modo a avaliar a sua libertação controlada a dois valores de pH diferentes (pH 5,6 e pH 2) e a melhorar a sua biodisponibilidade. As micropartículas foram preparadas por *spray drying* e caracterizadas relativamente ao tamanho das partículas e a morfologia da superfície. Métodos de cromatografia líquida de alta eficiência (CLAE) e espectrofotometria UV-Vis foram desenvolvidos para a detecção e quantificação analítica do ácido clorogénico. Ambos os métodos apresentaram uma resposta linear com coeficientes que correlação (R^2) melhores que 0,999 e bons resultados de precisão, com coeficientes de variação inferiores a 5%. Os limites de detecção (LOD) e quantificação (LOQ) por CLAE foram 0,19 $mg.L^{-1}$ e 0,64 $mg.L^{-1}$, respectivamente. Por espectrofotometria UV-Vis, os limites de detecção e quantificação foram

0,18 mg.L^{-1} (LOD) e 0,60 mg.L^{-1} (LOQ) em água a pH 5,6 e 0,09 mg.L^{-1} e 0,31 mg.L^{-1} em água a pH 2, respectivamente. Foi possível encapsular ácido clorogénico com alginato de sódio e quitosano modificado como agentes encapsulantes usando a técnica de *spray drying*. Foram obtidos rendimentos satisfatórios de 41,1% na produção de micropartículas de alginato de sódio e de 39,3% na produção das partículas de quitosano modificado. A distribuição em volume do tamanho das partículas revelou um tamanho médio de 2,8 µm e 3,2 µm para as micropartículas de alginato de sódio e quitosano modificado. Quanto à morfologia, as micropartículas apresentaram forma esférica com superfície lisa. Os estudos de libertação controlada do ácido clorogénico mostraram uma rápida libertação para os ambos os tipos de micropartículas em água em ambos os valores de pH. Usou-se CLAE e espectrofotometria de UV-Vis na avaliação da percentagem de libertação controlada do ácido clorogénico através de micropartículas de alginato de sódio e os resultados obtidos foram concordantes nas duas técnicas. No caso do quitosano modificado, 80% do 3-CQA foi libertado em aproximadamente 10 min. A taxa de libertação do ácido clorogénico (3-CQA) não foi significativamente afectada pelo valor de pH. Como primeiro estudo realizado, os resultados obtidos são significativos e reportaram o sucesso da microencapsulação do ácido clorogénico para aplicação em matrizes alimentares. Contudo, mais estudos devem ser realizados de modo a melhorar o padrão de libertação controlada do ácido clorogénico em produtos alimentares.

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Palavras-chave: Café, Ácido clorogénico, Microencapsulação, *Spray drying*, Alginato de Sódio, Quitosano modificado, Libertação controlada, Indústria alimentar

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Glossary

α-TP	α-tocopherol
β-CD NS	β-cyclodextrin nanosponges
3-CQA	3-caffeoylquinic acid
4-CQA	4-caffeoylquinic acid
5-CQA	5-caffeoylquinic acid
diCQAs	Dicaffeoylquinic acids
Abs	Absorbance
AU	Absorbance units
Ca^{2+}	Calcium ions
CAS	Chemical Abstracts Service
CGA	Chlorogenic acid
CQA	Caffeoylquinic acid
CS	Chitosan
CV	Coefficient of variation
DAD	Diode array detector
DD	Degree of deacetylation
EE	Encapsulation efficiency
FQAs	Feruloylquinic acids
HDI	Hexamethylene diisocyanate
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
LOD	Limit of detection

LOQ	Limit of quantification
NaCl	Sodium chloride
OSA	Octenyl succinic anhydride
PBS	Phosphate buffered saline
PTFE	Polytetrafluoroethylene
R^2	Correlation coefficient
RSD	Relative standard deviation
RT	Room temperature
SA	Sodium alginate
SEM	Scanning electron microscope
Tin	Inlet temperature
Tout	Outlet temperature
UV	Ultraviolet radiation
UV-Vis	Ultraviolet-visible

Chapter 1

Introduction

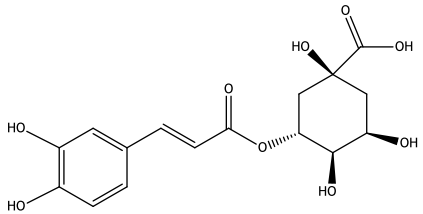
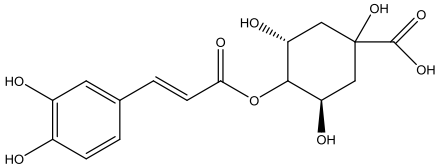
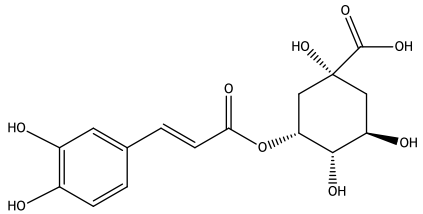
1.1 Chlorogenic acids

1.1.1. Overview and chemical composition

Chlorogenic acids (CGAs) are water soluble phenolic compounds found in many and diverse natural sources, such as plants, fruits and vegetables (Farah et al., 2008). They are *trans*-cinnamic acid derivatives naturally formed by the esterification of caffeic (3,4-dihydroxy-cinnamic acid), ferulic (3-metoxy,4-hidroxy-cinnamic acid) and *p*-coumaric (4-hidroxy-cinnamic acid) acids with (-)-quinic acid (Caprioli et al., 2013; Farah et al., 2008). Sunflowers, apples, pears, peaches, berries, prunes, sweet potatoes, lettuces and spinaches are some examples of where they natural occur (Cheng et al., 1995; Clifford et al., 2006; Nallamuthu et al., 2014) however green (or raw) coffee beans are known as the major source of CGAs (5–12 g/100 g) (Farah et al., 2008). CGAs are products of the phenylpropanoid pathway and act as protective agents in higher plants against environmental stress, such as infection, mechanical wounding and cell damage (Farah and Donangelo, 2006).

Caffeoylquinic acids (CQAs), dicaffeoylquinic acids (diCQAs) and feruloylquinic acids (FQAs) are the major CGAs classes in coffee (Duarte *et al.*, 2010), each one formed of at least three isomeric forms namely, three isomers of CQAs (3-, 4- and 5-CQA) (Table 1), three isomers of diCQAs (3,4-diCQA; 3,5-diCQA and 4,5- diCQA) as well as three isomers for FQAs (3-, 4- and 5-FQA). Amongst these, CQAs are considered as the main isomers of CGAs as 5-CQA is the most abundant one, comprising over 50% (w/w dry matter) of all CGAs in green coffee beans, followed by 3- and 4-CQA that comprise approximately 10% (w/w dry matter) (Narita and Inouye 2015).

Table 1 - Characteristics and chemical structures of caffeoylquinic acids (CQAs) present in coffee.

Compound (Abbreviation) *CAS number*	Synonym (IUPAC name)	Molecular Formula	Chemical Structure	Molecular weight ($g.mol^{-1}$)	Solubility in water ($mg.mL^{-1}$)
3-Caffeoylquinic acid (3-CQA) *327-97-9*	Chlorogenic acid ((1S,3R,4R,5R)-3-{[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-1,4,5-trihydroxycyclohexane-1-carboxylic acid)	$C_{16}H_{18}O_9$		354.31	3.44
4-Caffeoylquinic acid (4-CQA) *905-99-7*	Cryptochlorogenic acid (3R,4S,5R)-4-{[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-1,3,5-trihydroxycyclohexane-1-carboxylic acid)	$C_{16}H_{18}O_9$		354.31	3.12
5-Caffeoylquinic acid (5-CQA) *906-33-2*	Neochlorogenic acid (3-{[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-1,4,5-trihydroxycyclohexane-1-carboxylic acid)	$C_{16}H_{18}O_9$		354.31	3.44

* Adapted from *Hmdb* and *PubChem*, 2015.

1.1.2 CGAs in coffee beans

Coffee is among the most generally consumed commodity in the world and its composition has spread worldwide due to its unique sensory properties, as commercially, *Coffea arabica* and *Coffeea canephora var. robusta* are the most prominent species (El-Abassy et al., 2011).

Green coffee beans are seeds of the fruit of the coffee plant (coffee cherry). Each fruit contains two coffee beans enveloped by a thin seed skin known as coffee silver skin and an endocarp layer known as parchment. After this last, the ripe cherries are enveloped by a layer of pulp and, even more externally, by an outer red skin (Figure 1). Beans are the mainly responsible for full value and characteristic aroma of coffee once that all the bioactive compounds including, cellulose, minerals, sugars, lipids and polyphenols are presented there. Among them, CGAs are the most abundant polyphenol in coffee (Mussatto et al., 2011). Therefore, typical processing involves removing all the outer layers of the fruit, leaving only the beans for subsequent roasting (Narita and Inouye 2015). Raw coffee can hardly be defined as edible, and there are no claims of such material to produce a beverage, even if the active physiological component, caffeine, can be effectively extracted by hot water from crunched raw seeds. The grassy, astringent taste of such a brew is surely a deterrent against any commercial venture in that direction (Petracco et al., 2005). Roasted coffee bean is a globally consumed product which already embedded in the daily routine of many cultures, particularly in the north and south Americas, Japan and European Union countries (Farah and Duarte 2015).

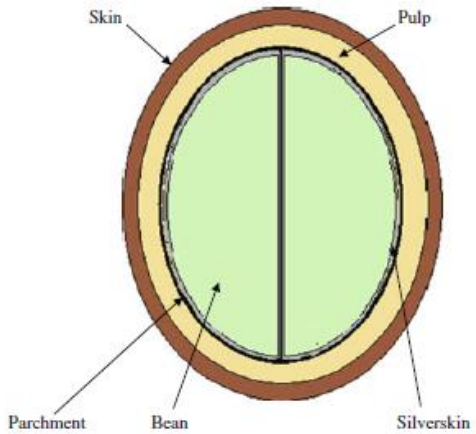


Figure 1 - Longitudinal cross-section of the coffee cherry. Adapted from Mussatto et al. 2011.

CGAs and subsequently CQA composition of coffee beans may vary from one species to another one, depending on different factors such as the climate conditions, nutricional value of the soil, the degree of ripeness as well as post-harvest and roasting process (Komes and Bušić 2014). From these factors that affect the coffee composition, genetic variability has been highlighted as it is directly contributing to the diversity in the acidity (Scholz et al., 2000). CGAs

are known to be the mainly responsible for the final acidity, astringency and bitterness of the coffee beverage (Ayelign and Sabally, 2013). Therefore, CGAs profile is typically used to identify and characterize the green coffee beans by species and their origin (Anthony et al., 1993; Ayelign and Sabally 2013; Bicchi et al., 1995). Depending on the species, the total CGAs present in coffee may vary from 6–7% to 14% dry basis (d.b.), with higher levels of CGA in Robusta coffee beans than in Arabica beans (Suárez-Quiroz et al., 2014). Although literature survey revealed that numerous production steps involved in coffee production such as bean roasting, freeze or spray drying, decaffeination and/or blending may influence the CGAs content. However due to the thermal instability of these phenolic compounds, the roasting process has been described as the most important step which may reduce the total CGAs content by up to 90% (Mills et al., 2013). High temperatures cause a breakage of the carbon–carbon bonds in CGA molecules, resulting in their isomerization and degradation. Longer periods of roasting result in higher isomerization and degradation and subsequently higher loss of CGAs. Besides that, other chemical transformations may also occur, such as the formation of a lactone ring (Figure 2) due to dehydration and formation of an intramolecular bond (Komes and Bušić 2014).

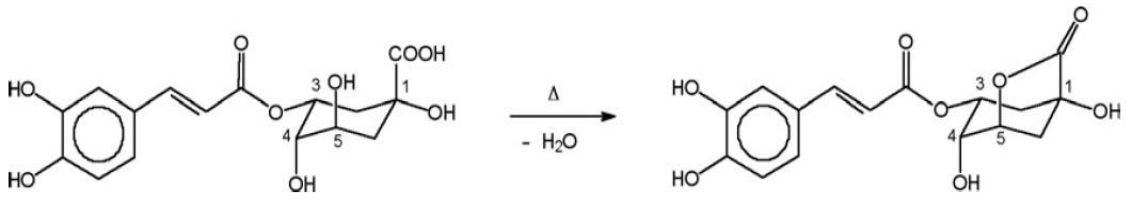


Figure 2 - Formation of a quinolactone from chlorogenic acid during roasting process. Adapted from Farah and Donangelo, 2006.

CGAs play an important role in the formation of pigments, taste and flavor of coffee beans, determining the end quality and acceptance of the related beverages. As it has been reported previously (Belay and Gholap 2009), there is a relation between the composition of the CGAs and the quality of the coffee beans. The decomposition of CGAs during roasting has contributed to significant changes not only in CGAs content, but also in sensory perception of the coffee beverage. Together with a complex combination of many other chemical compounds that are formed during the roasting process, decomposition of CGAs confers the beverage its characteristic flavor, and determines its final acidity, astringency and bitterness (Ayelign and Sabally, 2013). As a result of Maillard's reactions (chemical reaction normally between amino acids and sugars responsible for desirable flavor of browned foods), bitterness increases during roasting due to the release of caffeic acid as well as the formation of lactones and other phenol derivatives responsible for flavor and aroma (Farah and Donangelo 2006). Therefore, different

blend from various coffee species along with the different combination of treatments and processes that they undergo before reaching the consumer, may led to commercial coffee brands and ground coffee with different CGAs content (Farah and Donangelo, 2006; Ayelign and Sabally, 2013).

1.1.3 Interest and possible applications

Coffee as a complex matrix contains more than a thousand compounds which are appreciated not only for its taste but also for their stimulating properties. Among the various chemicals present in coffee, the biological effect of a few substances such as CGAs has been established. The high consumption of coffee in modern society has caused it to become one of the main dietary sources of CGAs and their inherent antioxidant activities, leading to a rapid expansion in interest in CGA research during the last decade. CGAs carry phenolic functions responsible for their antioxidant property. Figure 3 shows an example of an antioxidant mechanism presented by phenolic compounds.

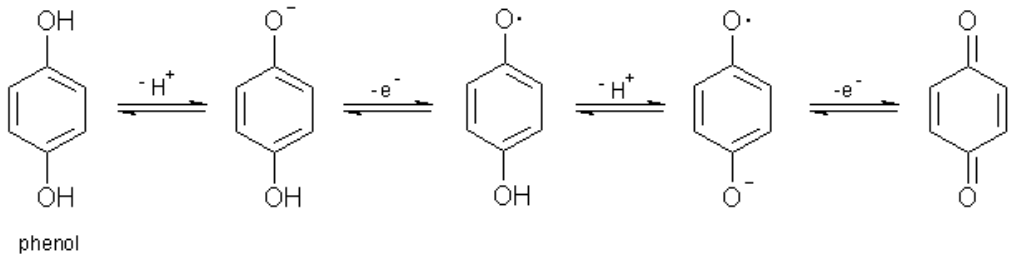


Figure 3 - Example of the antioxidant mechanism of Phenols. Adapted from ChemgaPedia, 2015.

Numerous epidemiological studies have frequently linked the consumption of a CGA-rich diet with numerous beneficial health effects, giving it an important role in the prevention of various diseases associated with oxidative stress, such as certain types of cancer, premature aging, strokes, Alzheimer's, Parkinson's, and other cardiovascular and neurodegenerative diseases (Belay and Gholap, 2009; Cano-Marquina et al., 2013). Data available in the literatures demonstrated that the consumption of green coffee extracts is attributed to an antihypertensive effect in rats and humans, improvement in human vasoreactivity, inhibition of fat accumulation and body weight in mice and humans, and modulation of the glucose metabolism in humans. These biopharmacological effects have been largely attributed to CGAs present in green coffee (Farah et al., 2008), more specifically to their known antioxidant properties, which are suggested to play an important role in protecting food, cells and any organ from oxidative degeneration. As antioxidants, CGAs have been suggested to reduce lipid

oxidation (Cano-Marquina et al., 2013). They have also been shown to have significant antiradical activity. Free radicals are highly reactive by-products of respiration, which when accumulated can cause severe damage to molecules and originate several diseases and premature aging. Green coffee bean extract containing CGAs, in particular 5-CQA, indicated the strongest antiradical activity. It has been reported that 5-CQA, can strongly react with hydroxyl free radicals, which may explain the neuroprotective effects of coffee consumption as it has been suggested by recent epidemiological studies (Yashin et al., 2013). In addition to their quelation and free radical scavenging abilities, CGAs are able to prevent molecule oxidation and damage (Nakatani et al., 2000, Laranjinha et al., 1994). Moreover and adding to their antioxidant power, CGAs have also been shown to potentiate other bioprotective effects, such as hepatoprotective activity in injured liver (Ji et al., 2013), and antibacterial and antiviral activities (Farah and Duarte, 2015).

As it was mentioned above, CGAs indicated various medicinal properties which lead to a growing interest in the dietary supplementation of food products with these compounds as natural antioxidants. Unfortunately, these valuable natural compound's applications are substantially limited by its low bioavailability and stability (Namallamuthu et al., 2014). CGAs can undergo enzymatic oxidation in many food processes and their instability in thermal processing of foods has also been reported (Namallamuthu et al., 2014). In addition, CGAs may suffer transesterification reaction which limits the product shelf-life during storage or distribution (Namallamuthu et al., 2014). The insufficient gastric residence time also proved to limit CGA's application once only a small proportion of its administration is absorbed. Furthermore, only one third of CGAs absorbed from gastrointestinal tract reaches blood circulation (Munin and Edwards-Lèvy, 2011).

Therefore, the administration of CGAs requires the formulation of a finished protecting product able to maintain their structural integrity until the consumption or the administration, mask its taste, increase its bioavailability and convey it towards a physiological target. Among the existing stabilization methods, microencapsulation has been widely reported as an alternative solution (Munin and Edwards-Lèvy, 2011).

1.2. Microencapsulation

1.2.1 Microcapsules: definition and characterization

Microencapsulation is the process in which a solid, liquid or gaseous component is surrounded by a coating of a continuous film of polymeric material. This conformation leads to the formation of capsules in general, microcapsules. These capsules offer a physical barrier

between the internal compound and the components from the surrounding environment providing higher protection against external factors such as oxygen, heat, humidity and light (Poshadri et al., 2010; Jyothi Sri et al., 2012). Microcapsules are small particles with a uniform wall around it, where the material inside the microcapsule is referred as the core, internal phase, or fill. The wall is called as shell, coating or membrane. Particle size of microcapsules may vary from 1 µm to 1 mm (Umer et al., 2011). Many morphologies can be produced for encapsulation, but there are two major morphologies: mononuclear and matrix type (Figure 4). Mononuclear microcapsules contain a single core enveloped by a shell, while in many cores are embedded in a matrix (Fang and Bhandari, 2010).

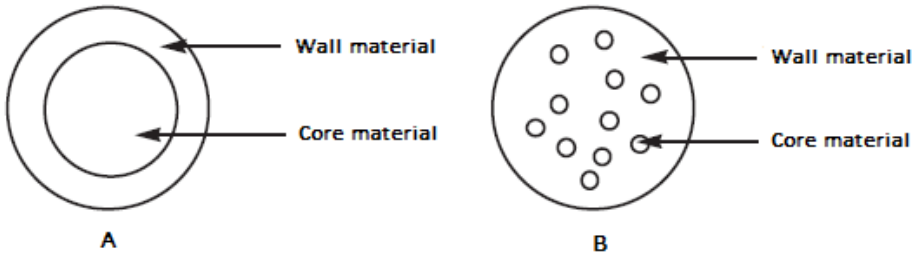


Figure 4 - Schematic representation of distinct structural microparticles types: (A) mononuclear; (B) matrix type. Adapted from Fang and Bhandari, 2010.

The specific shapes of microcapsules in different systems are influenced by the process technologies, and by the core and wall materials from which the capsules are made (Fang and Bhandari, 2010).

Microencapsulation can provide very interesting applications as it can (i) protect bioactive molecules and reduce the core reactivity with environmental factors; (ii) decrease the transfer rate of the core material to the outside environment; (iii) promote core substance handling; (iv) control core material release; (v) mask undesired core properties; (vi) convert liquid active components into a dry solid system, (vii) dilute the core material (viii) help the release of water insoluble substances into the aqueous means (Shahidi et al., 1993; Umer et al., 2011).

Indeed, microcapsules offer the possibility of controlled diffusion of lipophilic functional compounds and the possibility to promote better protection against lipid oxidation as well as better volatile retention, increasing the shelf-life of innumerous components. Permeability is another characteristic related with the purpose of microencapsulation. Microcapsules with impermeable walls are used in products where isolation of active substances is required, followed by a quick release under defined conditions. In addition, separation of reactive components, protection of sensitive substances, reduced volatility of substances, conversion of liquid ingredients into a solid state, taste and odor masking, as well as toxicity reduction can be

accounted as other objective of microencapsulation with impermeable wall materials. On the other hand, microcapsules with permeable walls enable prolonged release of active components into the environment, such as prolonged release of drugs, perfumes, deodorants and repellents (Jyothi Sri et al., 2012).

As it has been documented in the literature, microencapsulation has successfully been applied for a number of substances namely acids (e.g. chlorogenic acids) (Shi et al., 2006), oils (Frascareli et al., 2012), vitamins (Wilson and Shah, 2007), amino acids (Chen et al., 1992), essential oils (Martins et al., 2014), food coloring (Kandansamy and Somasundaram, 2012), enzymes (Anjani et al., 2007; Estevinho et al., 2014) and microorganisms (Islam et al., 2010).

During food processing, autoxidation of CGAs may occur which may results in the formulation of brown polymerized products. These drawbacks could be solved via microencapsulation of CGAs in order to improve its stability during the food processing for the reasons previously described.

1.2.2 Microencapsulation techniques

Microencapsulation of food ingredients into coating materials can be achieved by several methods. The selection of the microencapsulation process is governed by the physical and chemical properties of core, coating materials and the intended application of food ingredients (Poshadri et al., 2010). Table 2, schematizes several used methods for the preparation of microencapsulated products, as for food or other industries.

Table 2 - Microencapsulation techniques and each process steps.

Microencapsulation technique	Process steps	References
Spray drying	1. Disperse or dissolve active in aqueous coating solution; 2. Atomize; 3. Dehydrate. 4. Crosslink (optionally)	(Desai and Park, 2005) (Gouin, 2004) (Poshadri et al., 2010) (Wilson and Shah , 2007) (Zuidam and Nedovic, 2010)
Spray cooling/chilling	1. Disperse or dissolve active in heated lipid solution; 2. Atomize; 3. Cool.	(Desai and Park, 2005) (Gouin, 2004) (Poshadri et al., 2010) (Wilson and Shah, 2007) (Zuidam and Nedovic, 2010)
Spinning disk and Centrifugal coextrusion	1. Formation of core particles suspension in aqueous coating solution; 2. Thin film formation by suspension over a rotating disc; 3. Atomize;	(Gouin, 2004) (Poshadri et al., 2010)
Extrusion	1. Melt the coating; 2. Disperse or dissolve active in the coating; 3. Extrude; 4. Cool.	(Desai and Park, 2005) (Gouin, 2004) (Poshadri et al., 2010) (Wilson and Shah, 2007) (Zuidam and Nedovic, 2010)
Fluidized bed coating	1. Fluidize active powder; 2. Spray coating; 3. Dehydrate or cool.	(Desai and Park, 2005) (Gouin, 2004) (Poshadri et al., 2010) (Wilson and Shah, 2007) (Zuidam and Nedovic., 2010)
Coacervation	1. Prepare of emulsions with lipophilic active in oil phase; 2. Mix under turbulent conditions; 3. Induce three immiscible phases; 4. Cool; 5. Crosslink (optionally).	(Desai and Park, 2005) (Gouin 2004) (Martins et al., 2014) (Poshadri et al., 2010) (Wilson and Shah, 2007) (Zuidam and Nedovic,2010)

1.2.3 Microencapsulation techniques applied to food industry

Choosing the most adequate microencapsulation technique depends on the type of the active and encapsulating material, intended application and releasing mechanism. The basic difference between the existing methods relies in the relation between the active material (the core) and the encapsulate agent. Among the microencapsulation methods, the most commonly applied techniques in food industry are: spray drying, coacervation, fluidized bed coating, emulsification and inclusion complexation (Desai and Park, 2005; Gouin, 2004). Among these, spray drying has been the most commonly used techniques in food industry (Gouin, 2004, Martins et al., 2014).

1.2.4 Spray drying

Spray drying has been used in the food industry since the late 1950s and is known to be the most frequently technique applied to microencapsulation of polyphenols, providing protection against degradation/oxidation and converting liquids to powders (Gouin, 2004). This technique is a simple process, similar to one stage drying operation, which is capable of producing a wide variety of microcapsules at good yield, including microcapsules loaded with fragrance or flavor oils (Gharsallaoui et al., 2007).

The encapsulated active agent is homogenously dissolved into an aqueous solution containing the carrier material followed by, atomization under hot air flow. Subsequently, solvent evaporation results in the rapid solidification of the droplets. This technique is based in the pumping of the solution until it reaches the atomizer, which sprays the solution. Afterwards, obtained atomized materials are dried in the drying chamber under contacting with hot air at low pressure. Solid dry droplets are separated into the cyclone and collected in a subsequent container. Figure 5, schematizes the main process stages involved in spray drying (Braga, 2005; Wilson and Shah, 2007; Kissel et al., 2006).

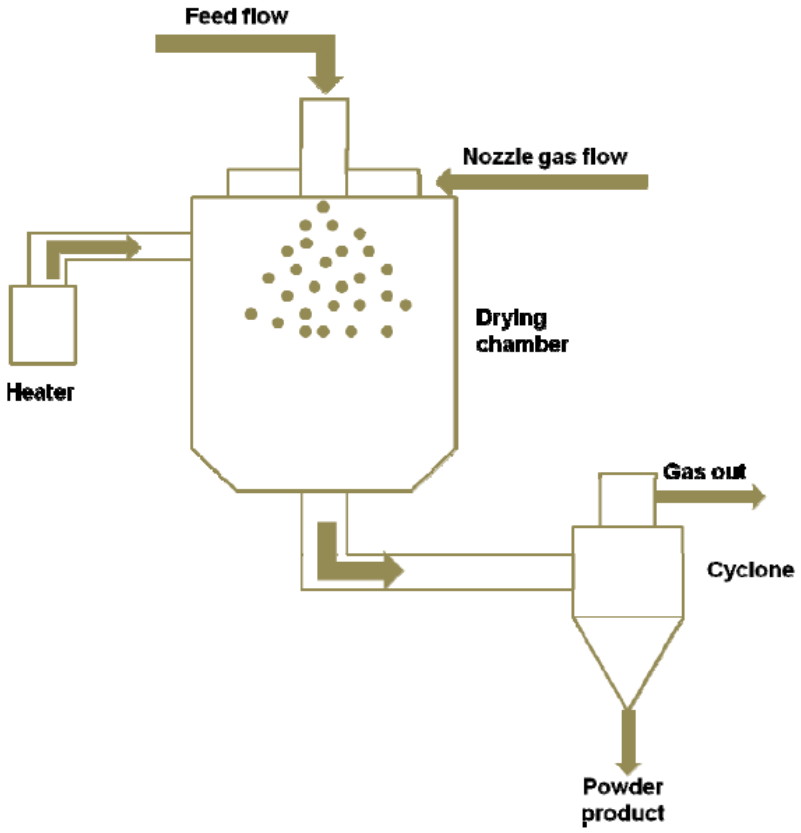


Figure 5 - Schematic ilustration of spray drying equipment. Adapted from Munin and Edwards-Lèvy, 2011.

The process variables are defined by the air temperature (leaving in and out of the system), air flow, temperature and humidity distribution, residence time and the geometry of the chamber (Kissel et al., 2006).

Spray drying is a simple and fast method, offering a low cost procedure and allowing obtaining a final product without the need of washing the microparticles in order to separate them from remaining solvent residues. The applied high temperatures do not come as an inconvenient when using thermos-sensitive compounds. The raised relation between specificsurface and the particles volume promotes the rapid evaporation of the solvent, in this circumstances the exposure time of the particles to the heat reduce and the temperature of the nucleus does not exceed the 100 °C, which reduces the possibility of undesired changes in the compounds properties (Desai and Park, 2005; Estevinho et al., 2013; Gharsallaoui et al., 2007; Gouin, 2004).

The type and concentration of the active and the encapsulating agent, velocity and the feed system temperature are important aspects in this method, influencing the microparticles characteristics (Silva et al., 2003).

1.2.5 Encapsulating agents

The composition of the coating material is the main determinant of the functional properties of the microcapsule and of how it may be used to improve the performance of a particular ingredient. The choice of the encapsulate agent depends on several factors, between them, in the process used for microcapsule formation and in the ideal release mechanism for each situation (Favaro-Trindade, 2008).

An ideal coating material should exhibit various characteristics, such as: easy workability during encapsulation; the ability to disperse the active substance; non-reactivity with the encapsulated material (compatible with core material); the ability to seal and hold the active material within its structure during processing (controlled release under specific conditions) and on prolonged storage; the ability to provide maximum protection to the active material against environmental conditions, such as oxygen (stabilization of core material); inert behavior toward active ingredients; the ability to provide an flexible, brittle, hard and thin coating; and finally, it may be abundantly and cheaply available (Goud and Park,2005; Poshadri et al., 2010; Jyothi Sri et al., 2012). After the desired coat functions and coating material restrictions have been established, a coating formulation can be developed from a range of available coating materials, modifiers, and solvents. Sophisticated shell materials and technologies have been developed,

and an extremely wide variety of functionalities can now be achieved through microencapsulation.

1.2.6 Biopolymers

Among the most commonly used materials as encapsulating agents, biopolymers have gained a much more attention due its natural origin, biocompatible, non-toxic, non-immunogenic and biodegradable properties (Stoica et al.,2013; Bu et al., 2006). The non-biodegradability of synthetic polymers has led to ecological problems (Sabiha-Hanim and Siti-Norsafurah, 2012) so the use of biopolymers for the encapsulation of polyphenols makes them attractive candidates for industrial applications (Stoica et al., 2013; Bu et al., 2006).

Biopolymers are macromolecules consisting of discrete building blocks naturally formed by microorganisms, plants and animals. These compounds are produced during the growth cycles of the mentioned organisms (Maleki, 2008; Pawar and Edgar, 2012; Rao, 2014) being chemically synthesized from biological starting materials such as amino acids, sugars and natural fats or oils (Stoica et al.,2013).

As an important source of polymeric materials, biopolymers have been shown a great potential for commercialization (Imre, 2013). They can modify the flow characteristics of fluids, stabilize suspensions, flocculate particles, encapsulate materials and produce emulsions. Consequently, they are now widely used as thickener, stabilizer, emulsifier, gelling agent and water-binding agents in the food, cosmetics, bioplastics and oil industries. Properties of biopolymers are dependent on the composition and molecular weight of the polymer (Imre, 2013; Jogdand, 2014). The structure of monomer used in polymer formation is directly effective on the properties that are required in different areas of work, such as: thermal stability, flexibility, good barrier to gases, good barrier to water, resistance to chemicals, biocompatibility and biodegradability (Güner et al., 2006).

The list shown below represents the biopolymers that have been normally applied as coating materials (Table 3). Starch, maltodextrin, gum arabic, pectin, chitosan and alginate are, among other common excipients, an example of natural polymers studied as matrix for microencapsulation. The representative biopolymers including proteins (such as albumin and gelatin) and polysaccharides (such as alginate and chitosan) have been the preferred polymers used in microencapsulation systems (Wang et al., 2006).

Table 3 - Biopolymeric materials commonly used for microencapsulation. Adapted from Desai and Park, 2005.

Category	Encapsulate agents	Widely used techniques	References
Carbohydrate	Starch*, Maltodextrins, Chitosan, Corn syrup solids, Dextran, Modified, Starch, Cyclodextrins, Sucrose, Modified Chitosan*, Sodium alginate*	Spray freeze or drying Extrusion Coacervation Inclusion Complexation	(Ascheri et al., 2003) (Muller, 2011) (Rocha et al., 2012) (Laohasongkram et al., 2011)
Cellulose	Carboxymethylcellulose*, Methylcellulose*, Ethylcellulose*, Celluloseacetate-phthalate, Celluloseacetatebutylate-Phthalate	Coacervation, Spray drying	(Greenberg and Mahoney, 1981) (Garcia et al., 1989) (Shahidi et al.,1993) (Uddin et al., 2001)
Gum	Gum acacia*, Agar, Carrageenan*	Spray drying, Coacervation	(Comunian, 2013) (Ascheri et al., 2003) (Kim et al., 2013) (Frascareli et al., 2012) (Dima et al., 2014) (Piacentini et al., 2013)
Lipids	Wax, Paraffin, Bees wax, Diacylglyerols, Oils, Fats, Phospholipids, Stearic acid	Emulsions, Liposomes, Film formation	(Kapusniak et al., 2006) (Shahidi et al., 1993)
Protein	Gluten, Casein, Gelatin*, Albumin, Peptides, Whey proteins hydrogel	Emulsion, Spray drying	(Betz and Kulozik, 2011) (Kim et al., 2013)

*Water soluble agents

1.2.6.1 Sodium Alginate (SA)

Alginate is one of the most naturally available polymers. This biopolymer is comprehensively used in food and beverages or pharmaceutical industries (Goh et al., 2012) due to its biocompatibility, low toxicity and low cost (Lee and Mooney, 2012). Besides that, they are also assumed to be non-immunogenic and biodegradable (Yang et al., 2011).

Alginate is a water soluble polysaccharide consisting of two basic building blocks, α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues, linearly linked together by 1-4 linkages (Figure 6) (Yoo et al., 2006; Goh et al., 2012).

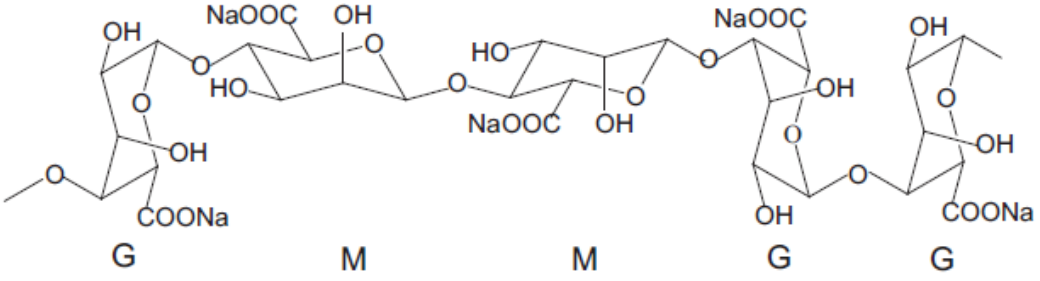


Figure 6 - Molecular structure of Sodium alginate. Adapted from Yang et al., 2011.

Depending on the alginate source, this linear conformation can be composed of consecutive G residues, consecutive M residues or alternating G and M residues (GM) (Lee and Mooney, 2012). Together with the copolymer composition (M and G contents), the units sequence and the molecular weight are also known to differ with the source of the alginate extract (Pawar and Edgar, 2012). Alginate extraction can be accomplished from algae as well as bacterial sources (Pawar and Edgar, 2012; Goh et al., 2012). For example, brown algae (*Phaeophyceae*), including *Laminaria hyperborea*, *Laminaria digitata*, *Laminaria japonica*, *Ascophyllum nodosum*, and *Macrocystis pyrifera* could be accounted as great sources for extraction of alginate. In order to precipitate the alginate, sodium or calcium chloride may be added to the extract (Lee and Mooney, 2012).

It should be stress that, approximately 30000 metric tons of alginate per year, is produced industrially (Pawar and Edgar, 2012). Its opens promising strategies for production of other biomaterials based on alginate which led to several commercial applications with different solubility, hydrophobicity and affinity for specific proteins (Pawar and Edgar, 2012). With regards to food industry, sodium alginate (SA) is used as a thickener and stabilizer in a wide range of products such as jelly, drinks (chocolate milk) and desserts (ice cream) (Goh et al., 2012). Furthermore, alginates coatings, as good oxygen barriers, are also known to retard lipid oxidation in foods (Kester and Fennema, 1997). Besides food industries, other applications such as ethanol production required alginate for encapsulation of Yeast cells (Goh et al., 2012). Cells immobilization, tissue engineering, drug delivery, controlled release and immobilization of micro-organisms are also some examples where alginates have been widely used as a type of desired biomaterial (Yang et al., 2011).

Due to the reactivity of its carboxylate side groups, alginate is known to form spontaneous gelation in the presence of divalent cations (such as calcium (Ca^{2+})) (Stoica et al., 2013). Calcium act as a crosslinker between the functional groups of alginate chains resulting in cross-linked alginate hydrogels that has been proved to be a great promise in biomedical applications. However, besides hydrogels induced by divalent cations, alginate can form acid gels at pH below the pKa value of 3.7 and 3.4 for G and M, respectively (Yang et al., 2011; Bu et al., 2006). Alginate gel structure is relatively stable at acidic pH, but it is easily swollen and disintegrated under mild alkali conditions. Thus, alginate gel has been applied to produce an effective controlled release carrier as forms of matrix, bead or microcapsules (Yoo et al., 2006).

Literatures suggest a temperature-dependent nature of alginates. At temperatures below the boiling point of water, non-covalent bonding between continuous polymeric segments keep the alginates intact so, thermostable alginate gels can be formed in the temperature range between 0 and 100 °C (Goh et al., 2012). Nevertheless, formation of thermostable alginate gel

up to 150 °C has been also reported in literature. Prolonged heat treatment at low or high pH may destabilize the gel.

As mentioned above, some uses of alginates depend on their thickening properties and their ability to increase the viscosity of aqueous systems using relatively low concentrations. The viscosity of alginate solutions is unaffected over the range of pH 5-11. Below pH 5, the free -COO^- ions in the chain start to become protonated, to -COOH, so the electrostatic repulsion between chains is reduced, they are able to come closer and form hydrogen bonds, producing higher viscosities. When the pH is further reduced, a gel will form, usually between pH 3-4. If the pH is reduced quickly from 6 to 2, a gelatinous precipitate of alginic acid will form. Above pH 11, slow depolymerization occurs on storage of alginate solutions, giving a fall in viscosity. Besides pH, alginate gels are also influenced by temperature once that viscosity decreases as temperature increases. Viscosity usually returns to a little less than the original value on cooling. However, if alginate solutions are kept above 50 °C for several hours, depolymerization may occur giving a permanent loss of viscosity. Alginate solutions can be frozen and defrosted without change of viscosity, as long as they are free of calcium.

1.2.6.2 Chitosan/Modified Chitosan

Another biopolymer commonly used as encapsulating material is chitosan (CS) (Stoica et al., 2013). Chitosan is a linear polysaccharide composed of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine (Figure 7) commercially obtained by alkaline deacetylation of chitin (Jolláes and Muzzarelli, 1999).

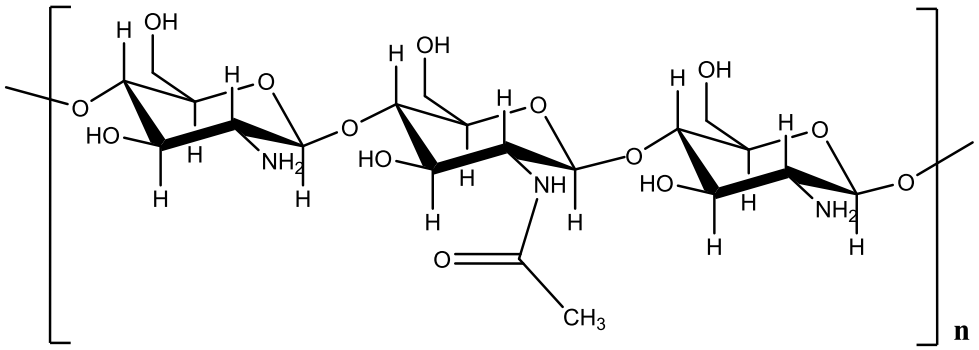


Figure 7 - Molecular structure of Chitosan.

Chitin is the most abundant nitrogen-bearing organic compound found in nature and is the second most available polysaccharide next to cellulose (Jolláes and Muzzarelli, 1999). The major sources of chitin production are the cuticles of crustaceans (shells of shrimp and crab) and exoskeletons of insects from where this polymer can be extracted by demineralization and

deproteinization using highly concentrated solutions of sodium hydroxide under high temperature (Dumitriu, 2002). The way how chitosan is prepared from chitin is determinant define important characteristics such as molecular weight, deacetylation degree and crystallinity (Estevinho et al., 2013). The degree of deacetylation (DD), determined by the proportion of D-glucosamine and N-acetyl- D-glucosamine, is the most important characteristic used to characterize both chitin and chitosan polymers. Therefore, chitin and chitosan are essentially the same polymer but with arbitrarily defined degrees of DD. Generally, if the DD is more than 40%, the term chitosan is used (Nordtveit, 1996). Together with the degree of deacetylation, molecular weight of this polymer (50-2000 kDa) has also a significant role in its biological properties (Estevinho et al., 2013).

Due to its biodegradability, biocompatibility, antimicrobial activity and non-toxicity, chitosan has been considered among the most valuable biopolymers for biomedical (artificial skin, wound dressing, contact lens) and pharmaceutical (medicine controlled release systems, capsules, microcapsules) applications. Regarding its antimicrobial activity, chitosan have also shown great promise to be used in food industry as preservative. The quality of a variety of food products have been insured by chitosan based films once that its activity can act against various microorganisms (Dutta et al., 2009). Nanoparticles, microspheres, hydrogels, films, and fibers are some examples of typical chitosan based forms used for industrial applications (Kumar, 2000).Although chitosan is an attractive biopolymer, it is a water insoluble material only soluble in an acidic aqueous medium (pH < 6) such as acetic, nitric, formic, latic, hydrochloric, perchloric and phosphoric acids (Kurita, 2006; Rinaudo, 2006; Sankararamakrishnan and Sanghi, 2006). Its pH-sensitive behavior, given to the large quantities of amino groups on its chain, provides chitosan the ability to dissolve easily at low pH (acidified medium) but hardly at higher pH ranges, which limit its application to bioactive agents such as drug carrier (Estevinho et al., 2013). This mechanism of pH sensitive swelling under low pH conditions involves the protonation of amine groups of chitosan (Figure 8).

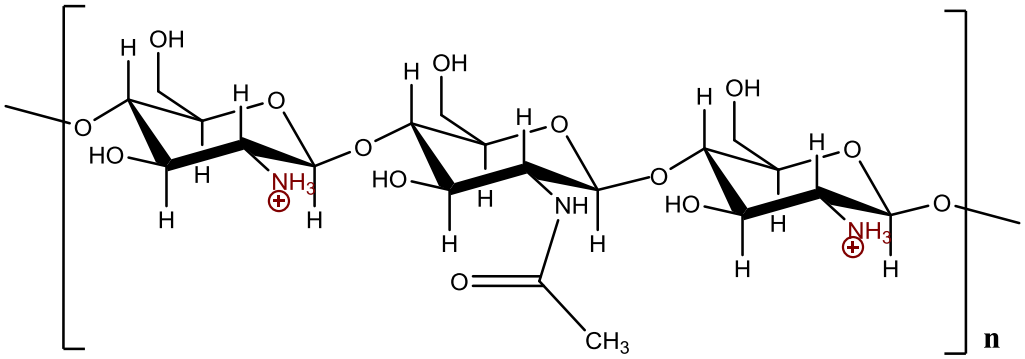


Figure 8 - Chemical behavior of Chitosan under low pH conditions.

However, chitosan can undergo chemical modification very easily. This property is attributed to the presence of free amino groups, which increases the reactivity of the polymer. Therefore, it can be readily modified by reactions at the amino groups, allowing the improvement of chemical and mechanical properties of chitosan. These chemical modifications would not change the fundamental skeleton of chitosan and would keep the original physicochemical and biochemical properties while bringing new or improved properties (George, 2006). The main goals of modifying chitosan chemically are to provide derivatives that are soluble at neutral and basic pH values, to control hydrophobic, cationic, and anionic properties as well as to attach various functional groups and ligands. This advantage is useful for drug carriers and for food industrial applications.

Into what comes to microencapsulation, chitosan has one important advantage over other encapsulating agents, which is the possibility to establish covalent or ionic bonds with the crosslinking agents, building a network, where the active substance stays retained. Consequently, these chemical bonds carry advantages in terms of controlled release. The crosslinking reaction is mainly influenced by the size and type of crosslinker agent and the functional groups of chitosan, the smaller the molecular size of the crosslinker, the faster the crosslinking reaction, since its diffusion is easier. Depending on the nature of the crosslinker, the main interactions forming the network are covalent or ionic bonds. Covalently crosslinked hydrogels present the crosslinking degree as the main parameter influencing important properties such as mechanical strength, swelling and drug release. Such gels generally exhibit pH sensitive swelling and drug release by diffusion through their porous structure. Having this, hydrogels based on covalently and ionically crosslinked chitosan can be considered as good candidates, e.g., for the oral delivery of drugs (Estevinho et al., 2013; George, 2006).

To the best of our knowledge, limited studies have been performed with regards to the encapsulation of chlorogenic acids. For this purpose and to overcome the limitations of chlorogenic acid for industrial applications (e.g. food industry), the present study aims the encapsulation of chlorogenic acid (3-CQA) by spray drying into biopolymer-bases microparticles. The development and characterization of a sustained-release system for chlorogenic acid (3-CQA) through two natural biodegradable polymers (sodium alginate and modified chitosan) together with the study of their release profiles under different pH conditions (pH 5.6 and pH 2) are intended. Validation of an analytical method for 3-CQA quantification and characterization of the obtained microparticles in terms of size and shape are also pretended.

Chapter 2

State of the Art

In the last few years, formulations of new functional food products containing natural compounds have shown an increased growing interest due to their antioxidant activity and other beneficial health effects. However, low stability, low bioavailability and the unpleasant taste presented by certain compounds, such as polyphenols, have restricted many of their industrial applications. In order to overcome such limitations, many encapsulation methods have been described in the literature, among which some have been successfully applied to polyphenols. Nevertheless, there are limited studies for encapsulation of polyphenolic compounds present in coffee, in particular for chlorogenic acids (CGAs) (Table 4).

Carvalho et al., 2013 investigated the process of microencapsulation of green coffee oil (*Coffea arabica*) using emulsions stabilized by lecithin and lecithin-chitosan in order to improve the oxidative stability and maintain the sun protection factor of the oil, for possible cosmetic applications. The obtained microparticles produced by spray drying used corn syrup and two chemically octenyl succinic anhydride (OSA) modified starches as wall materials and were characterized for particle size distribution, microstructure, encapsulation efficiency (EE) and *in vitro* sun protection factor. Regarding to EE, values ranged from 86 to 97 % and was significantly influenced by the kind of wall material and the use of chitosan. Microparticles prepared with modified starch or combined with corn syrup (50:50) presented the best encapsulation efficiency values. The ones formed by the mixture of two modified starches with corn syrup presented better EE just when stabilized by lecithin– chitosan. Chitosan presented to contribute to a greater stability of the emulsions, providing lower surface oil content in microparticles and, consequently, higher values of EE. The particle mean diameter varied from 14.51 µm to 29.19 µm, being 14.51 µm to 19.50 µm for emulsions stabilized by lecithin–chitosan and 16.40 µm to 29.19 µm for emulsions stabilized by lecithin only. Microparticles produced with modified starch and stabilized by lecithin–chitosan presented larger diameter with the addition of corn syrup than microparticles produced only with modified starch. In general, all the particles exhibited spherical shape and irregular surface. However, some cracks and holes were observed for syrup microparticles compromising and reducing, by the enhancement of air permeability, their ability to protect the active material. No cracks or holes were referred to microparticles of modified

starch. In terms of oxidative stability, the highest values were obtained for microparticles produced with modified starch and for the combination of it with corn syrup. Microparticles prepared with lecithin-chitosan also exhibited higher oxidative stability when compared to the particles constituted just by lecithin. The presence of chitosan in microparticles proved to influence positively the oxidative stability once it can provide better barrier to oxidation.

The influence of different combinations of wall materials used for microencapsulation of green coffee oil by spray drying was also studied by Silva et al., 2013 In their study, microparticles were prepared using modified starches or gum arabic, with maltodextrin, and their characterization was also related to encapsulation efficiency, particle mean diameter, *in vitro* sun protection factor and oxidative stability. Encapsulation efficiencies varied from 82% to 99% and, for all the combinations of wall materials, values increased with the use of homogenization pressure in the emulsion preparation step. Particles mean size (10.7 µm – 16.0 µm) and oxidative stability presented no variation with the use of homogenization pressure but they were influenced by the type of combination of wall material. As in Carvalho et al., 2013 study, the obtained microparticles presented spherical shape and various sizes, which is a typical characteristic of spray dried powders. The lack of wall fissures or porosity on the particles surface indicates complete coverage of the wall material over the core, confirming the high EE values. In general, microparticles revealed higher oxidative stability than pure green coffee oil. Thereby, the microencapsulation process using modified starches with maltodextrin has promoted higher stability of the oil. Regarding the oxidative stability and EE, the highest response was obtained from microparticles made by the mixture modified starch and maltodextrin.

Besides corn syrup, modified starch and maltodextrin, gum arabic was individually used as encapsulating agent for microencapsulation of roasted coffee oil. Frascareli et al., 2012 evaluated the influence of process conditions on the microencapsulation of coffee oil (*Coffea arabica*) by spray drying using gum arabic and oil concentration (10%-30%) was the variable that most affected encapsulation efficiency. However, total solid concentration (10%-30%) and inlet air temperature (150-190 °C) also showed some influence in the obtained results. Both, encapsulation efficiency and oil retention were negatively influenced by oil concentration and inlet air temperature, and positively affected by total solid content. The higher the oil concentration, the lower the encapsulation efficiency and the same behavior was observed for the inlet air temperature. Higher the oil concentration the lower amount of gum Arabic available to provide a structural matrix to keep the oil droplets encapsulated, decreasing the encapsulation efficiency. The decrease in the encapsulation efficiency with the increase of temperature could be related to the fact that high temperatures promote a faster drying of the

external area, as compared to the internal one, creating cracks in the particle wall, resulting in oil release. On the other hand, particles produced with higher solid content presented higher encapsulation efficiency. Encapsulation efficiency varied from 48% to 82% and the best result was reported to particles produced from emulsions with higher solid content (25%-30%), lower oil concentration (10%-15%) and lower drying temperatures (150-170 °C). Besides encapsulation efficiency, also particle size distribution can be influenced by the conditions previously mentioned. The increase in total solid content resulted in larger particle size, while the increase in the oil concentration resulted in smaller particles. This can be explained by the viscosity of the feed emulsion, which increased with solid concentration and decreased with the increase of oil concentration. Higher the emulsion viscosity, larger are the atomized droplets and, therefore, larger the powder particles. Particle mean diameter varied from 7.88 µm to 13.13 µm and their evaluation with respect to oxidative stability showed good results during storage at room temperature (25 °C), but not at 60 °C. However, at this temperature, pure oil presented higher lipid oxidation than encapsulated, confirming the protective effect of microencapsulation on the oxidative stability of the oil and, consequently, in the increase of it shelf-life.

Regarding the few studies about microencapsulation of chlorogenic acid, different wall materials and different microencapsulation techniques were suggested until now (Table 4). Ramírez-Ambrosi et al., 2014 for example, evaluated β-cyclodextrin nanosponges (β-CD NS) as encapsulating agent for inclusion complexation of polyphenols found in apples, where chlorogenic acid was included. In their study, the highest encapsulation efficiency of chlorogenic acid (77.5%) was obtained with nanosponges of hexamethylene diisocyanate (HDI) in a 1:8 ratio and *in vitro* dissolution studies confirmed the hydrophilic behavior of the this polyphenolic compound. Taking into account the results, chlorogenic acid was successfully encapsulated by β-CD NS with a high degree of retention and protection, enhancing it availability for formulation of functional foods or food supplements.

On the other hand, the ability to form complexes of chlorogenic acid with amylose using conventional hydrothermal method was also investigated by Lorentz et al., 2012. Liophilization was used in order to improve encapsulation method and, once that was required heating to 90 °C to form complexes, chlorogenic acid and its derivative (4-O-palmitoyl chlorogenic acid) were, among others, evaluated for thermal stability through UV-Vis spectrophotometry. The UV-vis spectra before and after heating to 90 °C revealed that no change was observed, proving that chlorogenic acid was stable at this temperature. As similar results were obtained for 4-O-palmitoyl chlorogenic acid, amylose complexation formation proved to be suitable for chlorogenic acid and for 4-O-palmitoyl chlorogenic acid encapsulation.

Yeast cells (*Saccharomyces cerevisiae*) were another option presented by Qi et al., 2010 to encapsulate chlorogenic acid. The optimal encapsulation was obtained for 6 h at 40 °C and 3:1 core material/wall material ratio (g/g) in 6 mL of distilled water and corresponded to a maximum encapsulation efficiency of 18.9%. Infrared spectral analysis indicated the disappearance of characteristic function groups of chlorogenic acid and fluorescence microscopic studies revealed that the microcapsules were spherical in shape. The similar retention time and UV-Vis profiles between unmicroencapsulated and microencapsulated chlorogenic acid were found through HPLC analysis and results suggested that chlorogenic acid was successfully encapsulated and no significant chemical changes occurred during the encapsulation process.

Studies concerning the potential of encapsulation for controlled delivery of CGAs are even less than those reported just for its microencapsulation. Nallamuthu et al., 2014 and Shi et al., 2007 were, to the best of our knowledge, the only authors reporting studies with this purpose. As Qi et al., 2010, Shi et al., 2007 also investigated microencapsulation of chlorogenic acid (CGA) in yeast cells (*S. cerevisiae*). In their study, the obtained microparticles were tested for *in vitro* release of CGA and the release profile of yeast encapsulated chlorogenic acid was evaluated in HCl (pH 1.2), in double-distilled water and in phosphate buffer (pH 7.4) in order to simulate stomach and intestine conditions, respectively. Results showed that the release rate of chlorogenic acid in simulated gastric fluid (pH 1.2) was the highest, followed by the phosphate buffer (pH 7.4). Besides that, release profiles also showed that more than 95% of chlorogenic acid was released within 2 h in HCl, and the complete release was achieve after 5 h.

In Nallamuthu et al., 2014 study, chlorogenic acid (CGA) was microencapsulated into chitosan nanoparticles by ionic gelation method and exhibited, through scanning electron microscopy (SEM), a size of 210 nm and a regular, spherical shape distribution. The release profile of CGA from the obtained chitosan nanoparticles was investigated at 37 °C over a period of 100h and 0.1 M HCl and PBS were also used to simulate stomach and intestine conditions. Results revealed a controlled release pattern characterized by a fast initial release (25%) during the first 10 h, followed by slower and continuous release (69%) till 100 h. In the same period of time, the release of CGA was faster in PBS than in HCl.

As it is possible to see, all the studies reported for microencapsulation of chlorogenic acid used different microencapsulation techniques and different wall materials. Different results presented for morphology and particle size, encapsulation efficiency and controlled release can be related with these differences. Considering the interest of sodium alginate and chitosan as encapsulating agent (Chapter 1) and spray drying as encapsulation technique, the study of the

release of chlorogenic acid from sodium alginate and modified chitosan microparticles prepared by spray drying prove to be very important.

Table 4 - Overview on microencapsulation techniques applied to chlorogenic acids.

Compound	Compound origin	Microencapsulation technique	Wall material	Encapsulation efficiency (EE) (%)	Particle size	Particle shape	Analytical method	Results	References
Chlorogenic acid (CGA)	n.a.	Ionic gelation	Chitosan	59	~250 nm	Spherical	HPLC	-Controlled release profile and preserved antioxidant activity under *in vitro* conditions; - Higher heat stability; - Better delivery of CGA. - Shape of nanoparticles presented to be homogeneous and with smooth surface.	Nallamuthu et al. 2014
Chlorogenic acid (CGA)	Apple	Inclusion complexation	β-CD nanosponges	78	n.a.	n.a.	UV-Vis spectrophotometry	-Successful encapsulation of chlorogenic acid within β-CD NS (high degree of retention and protection); -Enhancement of CGA bioavailability.	Ramírez-Ambrosi et al.2014
Chlorogenic acid (CGA)	n.a.	n.a.	*S. cerevisiae* cells *	19	n.a.	Spherical	HPLC UV-Vis spectrophotometry	- Successful encapsulation of CGA; - No significant chemical changes on CGA.	Qi et al. 2010
Chlorogenic acid (CGA)	n.a.	Freeze-drying	*S. cerevisiae* cells	≤13	n.a.	n.a.	HPLC-DAD	-Yeast encapsulation (EE %) enhanced by plasmolysis off cells and increased CGA purity (5, 85, and 98 %); -Encapsulation increased : (1) Storage stability of CGA; (2) CGA release: Simulated Gastric Fluid (SGF)> phosphate buffer > water.	Shi et al. 2007
Neochlorogenic acid and 4-O-palmitoyl chlorogenic acid	n.a.	Liophilization and amylose complexation	Amylose	n.a.	n.a.	Variable	UV spectrophotometry	-Thermal stability of CGA (90 °C); -Thermal stability of 4-Opalmitoyl chlorogenic acid (90 °C); -No alteration or hydrolyzation of 4-Opalmitoyl chlorogenic acid during amylose complex formation; - Effective interactions between amylose and 4-C-palmitoyl chlorogenic acid.	Lorentz et al. 2012

n.a.: Not available; **β-CD:** β-cyclodextrin; **HMDI:** Hexamethylene diisocyanate; **HPLC:** High Performance Liquid Chromatography.

Table 4 – Overview on microencapsulation techniques applied to chlorogenic acids.

Compound	Compound origin	Microencapsulation technique	Wall material	Encapsulation efficiency (EE) (%)	Particle size	Particle shape	Analytical method	Results	References
Green coffee oil	*Coffea arabica*	Spray drying	(1) Corn syrup; (2) Modified starch; (3) Combination of corn syrup and modified starch;	86 - 97	14.51 - 29.19 µm	Spherical	UV-Vis spectrophotometry	Microparticles : (1) Corn syrup: holes and cracks; lower oxidative stability; (2) Modified starch: spherical shape without cracks or holes; - Modified starch (only) and corn syrup with modified starch: higher oxidative stability; - Emulsions stabilized by lecithin and chitosan showed higher stability; - Particles exhibited spherical shape and irregular surface.	Carvalho et al. 2013
Green coffee oil	n.a.	Spray drying	(1)Combinations of modified starches with maltodextrin; (2) Gum arabic with maltodextrin.	82 - 99	10.70 - 16.00 µm	Spherical	HPLC	- High pressure homogenisation increased encapsulation efficiency; - Type of wall material influenced oil retention; -Modified starches and maltodextrin presented the highest oxidative stability.	Silva et al. 2013
Roasted coffee oil	*Coffea arabica L.*	Spray drying	Gum arabic	48 - 82	7.88 - 13.13 µm	Spherical	n.a.	- Rough and smooth surfaces were observed for the obtained particles; - Particles showed to be stable at room temperature (25 °C); - At 60 °C, pure oil presented higher lipid oxidation than encapsulated, confirming the protective effect of microencapsulation on the oxidative stability. -Microencapsulation increased oil shelf-life;	Frascareli et al. 2012

n.a.: Not available; **β-CD:** β-cyclodextrin; **HMDI:** Hexamethylene diisocyanate; **HPLC:** High Performance Liquid Chromatography.

Chapter 3

Material and Methods

3.1 Materials

3.1.1 Standards and Reagents

All reagents used were of analytical grade purity. Acetonitrile (HPLC gradient grade) was obtained from VWR (Prolabo) (Leuven, Belgium). Citric acid was supplied from Merck (Germany). Filtered water used for HPLC analysis was prepared by vacuum purification through 0.45 µm Nylon 66 filter membranes.

Chlorogenic acid standard (3-caffeoylquinic acid, $C_{16}H_{18}O_9$, CAS: 327-97-9) (≥ 98% purity) was acquired from Sigma-Aldrich Chemical Co. (MO, USA). Sodium alginate (alginic acid, sodium salt) (180947-100g) was obtained from Sigma Aldrich Chemical Co. (USA) and modified chitosan (pharmaceutical grade water-soluble chitosan) from China Eastar Group (Dong Chen) Co., Ltd. (Batch no. SH20091010). Modified chitosan (water-soluble) was produced by carboxylation and had a deacetylation degree of 96.5% and a viscosity of 5mPa.s^{-1} (1%, 25 °C).

Deionised water was obtained in the laboratory using MilliporeTM water purification equipment (Massachusetts, USA).

3.1.2 Standards preparation

For HPLC analysis, a stock solution with a concentration of 400 mg.L^{-1} of chlorogenic acid (3-CQA) was prepared by adding 4 mg of 3-CQA into 10 mL of distilled water. The resulting solution was used to perform 12 calibration standards ranging concentration levels from 1 to 400 mg.L^{-1}.

Since to obtain a linear response it was necessary that the absorbance values were not above 1 AU, a new stock solution with a concentration of 15 mg.L^{-1} of chlorogenic acid was prepared by adding 0.75 mg of 3-CQA in 50 mL of distilled water. Therefore, the absorbance of the new calibration curve was within the range of UV-Vis spectrophotometer. For this purpose, 7 calibration standards were prepared in a concentration range of 0.5-15 mg.L^{-1}. Standards and stock solutions were stored at -22 °C until analysis.

Validation of both analytical methods was investigated through parameters such as linearity, limit of detection (LOD), limit of quantification (LOQ), precision (intra-day and inter-day) and sensitivity.

3.1.3 Equipment

The instrumental analysis of chlorogenic acid (3-CQA) was performed on Merck Hitachi Elite LaChromatogragh (Tokyo, Japan) with a quaternary system of pumping (L-2130) which is equipped with LiChroCART® RP-18 end-capped (250 mm x 4 mm, 5 µm) column attached to a guard column (4 mm x 4 mm, 5 µm) of the same type and L-2200 auto sampler with L-2455 UV-Vis spectrophotometry diode array detector. EZChrom Elite 3.1.6 software was used for data acquisition and peak integration. The pH of mobile phase was controlled by a pH meter (pH Meter GLP 21, Crison).

A spectrophotometer UV-Vis V-530 (Jasco) was also used for analysis of 3-CQA. Microencapsulation was performed using a BÜCHI B-290 spray dryer (Flawil, Switzerland).

3.2 Methods

3.2.1 High Performance Liquid Chromatography (HPLC)

Detection and quantification of loaded chlorogenic acid (3-CQA) in water at normal pH (5.6) was performed isocratically through a Merck Hitachi Elite LaChromatogragh (section 3.1.3) with a mobile phase composed by 90 % eluent A: 10 mM citric acid solution (acidity adjusted to pH 2.6) and 10% eluent B: acetonitrile. The injection volume was 10 µL and the analysis was performed with a flow rate of 1 $mL.min^{-1}$ at room temperature, over 17 min. The maximum absorbance for the analyzed compound was 325 nm.

Distilled water was filtered through 0.45 µm nylon filter membranes and both phases were degassed with helium (99.995%) over 30 min (each liter). The same was done for all washing solutions.

3.2.2 UV-Vis Spectrophotometry

Due to the clog of the chromatographic column during detection and quantification of the loaded chlorogenic acid from sodium alginate microparticles, UV–Vis spectrophotometry was used as another method for quantitative analysis of this compound. The amount of loaded chlorogenic acid was determined in water at two different pH values (pH 5.6 and 2.0). For that, two high precision cells of quartz (Hellma Analytics) were used. Before measurement, each one of these two cells was filled with 1 mL of water with pH 5.6 or pH 2, depending on the case, and

an auto zero was performed. One of the cells was kept as reference (with water) till the end of the measurement. The other one was used to analyze the amount of loaded chrologenic acid in each sample.

Considering the maximum absorbance wavelength from the spectrum, absorbance detection was done at 323 nm and 324 nm for pH 5.6 and pH 2, respectively. All the analyses were performed at room temperature.

3.2.3 Preparation of chlorogenic acid microparticles: Spray drying process

For this purpose, a stock solution containing chlorogenic acid was prepared in concentration of 10 $g.L^{-1}$ and was well homogenized in ultrasound bath for 5 min. Chlorogenic acid solution was sealed with parafilm and kept at -20 °C until the moment that it was necessary to prepare the microparticles. In addition, the encapsulating agent (1 g) were placed in glass bottle along with 100 mL of deionized water and let to dissolve well under continuous stirring at room temperature. To ensure full dissolution of the polymer molecules, each encapsulating agent solution was kept under stirring (500 rpm) at room temperature, 24 hours before start the encapsulation process. The concentration of encapsulating solution was 10 $g.L^{-1}$ for modified chitosan and sodium alginate. Chlorogenic acid solution (2 mL, 10 $g.L^{-1}$) was added and mixed with 100 mL of each encapsulating agent solution at constant agitation speed (500 rpm) and at room temperature during 30 min before start the spray drying process. Microencapsulation was separately performed using a spray-dryer BÜCHI B-290 (Flawil, Switzerland) with a standard 0.5 mm nozzle. Table 5 presents the experimental conditions used for spray-dryer. The outlet temperature (Tout) is a consequence of the other experimental conditions and also of the solution properties and was around 67 °C. The same conditions were used for both types of the prepared microparticles. Microparticles were collected and stored in falcon tubes at 4 °C for further analysis.

Table 5 - Experimental conditions of spray drying conditions process.

Spray-dryer parameters	Values
Tin (°C)	115
Tout (°C)	67
Solution flow rate (%)	15
Aspiration (%)	100
Nozzle (mm)	0.5
Pressure (bar)	6

3.2.4 Characterization of chlorogenic acid microparticles

The obtained microcapsules were characterized in terms of particle size (μm), shape and product yield (%).

3.2.4.1 Product yield (%)

Product yield (%) was separately measured for each microencapsulation experiment and it was expressed as the ratio of the mass of powder (microparticles) obtained at the spray-dryer output and the solid content of the initial infeed solution.

3.2.4.2 Particle size distribution

The size distribution of chlorogenic acid (3-CQA) microparticles produced with sodium alginate and modified chitosan were individually evaluated by laser granulometry using a Coulter Counter-LS 230 Particle Size Analyser (Miami, FL, USA) equipped with small volume plus. Each small powder sample was dispersed in ethanol (Aga, 96%) and ultrasound-irradiated over 10 s to avoid particles agglomeration. Characterization was done by number and volume average and the results were obtained as an average of two 60 s runs.

3.2.4.3 Scanning Electron Microscopy (SEM) analysis

The morphological analysis of the obtained microparticles was performed by Scanning Electron Microscopy, SEM (Fei Quanta 400 FEG ESEM/EDAX Pegasus X4M). Samples were coated with gold by pulverization under vacuum in a Jeol JFC 100 apparatus and analyzed by SEM for surface structure observation. All analyses were performed at Centro de Materiais da Universidade de Porto (CEMUP).

3.2.5 Controlled release studies of chlorogenic acid (3-CQA)

The release profile of chlorogenic acid (3-CQA) from microparticles prepared with two different wall materials (sodium alginate and modified chitosan) was investigated in aqueous solutions at two different pH values (5.6 and 2). HCl was added to distilled water under continuous stirring until achieve the pH around 2.0. Another solution for studying the controlled release was normal distilled water with a pH of 5.6. Microparticles (3 mg) were suspended in 1.5 mL of aqueous solution at pH 2.0 or 5.6 being continuously stirred (60 rpm) with a small magnet at room temperature. Samples were done in duplicated and were measured after defined time intervals: 0 min, 1 min, 2 min, 5 min, 10 min, 20 min, 30 min, 45 min, 1h, 2h, 4h, 6h and 24h. The released amount of chlorogenic acid was also evaluated in duplicate over the specific time using HPLC-DAD or UV-Vis spectrophotometry methods.

With regards to HPLC-DAD, samples were injected directly after two times filtration through 0.45 µm and 0.22 µm PTFE filters (OlimPeak, Teknokroma). Filtered solutions were analyzed according to chromatographic conditions explained previously.

Samples analyzed by UV-Vis spectrophotometry were prepared at the same time intervals. However, in order to keep the absorbance of the real sample in the absorbance range of the UV-Vis spectrophotometry (0-1 AU) samples were prepared with 3 mg of microparticles in 4.5 mL of aqueous solution at two different pH (5.6 and 2). 1 mL of the prepared samples was analyzed using UV high precision cells made of quartz (Hellma Analytics). Each analysis was performed at 323-324 nm and room temperature, over 30 s.

Chapter 4

Results and Discussion

4.1. Validation of analytical method

The validation of both analytical methods (HPLC-DAD and UV-vis spectrophotometry) was done in order to ensure their suitability for chlorogenic acid quantification and to allow the study of the chlorogenic acid controlled release profiles. Analysis of chlorogenic acid by HPLC was performed in duplicated in accordance to the method described and validated by Moeenfard et al., 2015.

Validation performance parameters like precision (intra-day and inter-day), sensitivity as well as the limits of detection (LOD) and of quantification (LOQ) were determined.

4.1.1. HPLC-DAD analysis

For HPLC-DAD analysis, calibration curves were constructed by plotting the peak area (mAU) against the corresponding concentrations ($mg.L^{-1}$) by duplicate injections of 10 µL of chlorogenic acid standard solutions at twelve concentration levels between 1 and 400 $mg.L^{-1}$ (1, 2, 5, 10, 25, 50, 75, 100, 150, 200, 300 and 400). Figure 9 presents the chromatogram of chlorogenic acid (3-CQA) obtained in the present study. The retention time of the analyte under the referred conditions (section 3.2.1) was 12.80 ± 0.17 min.

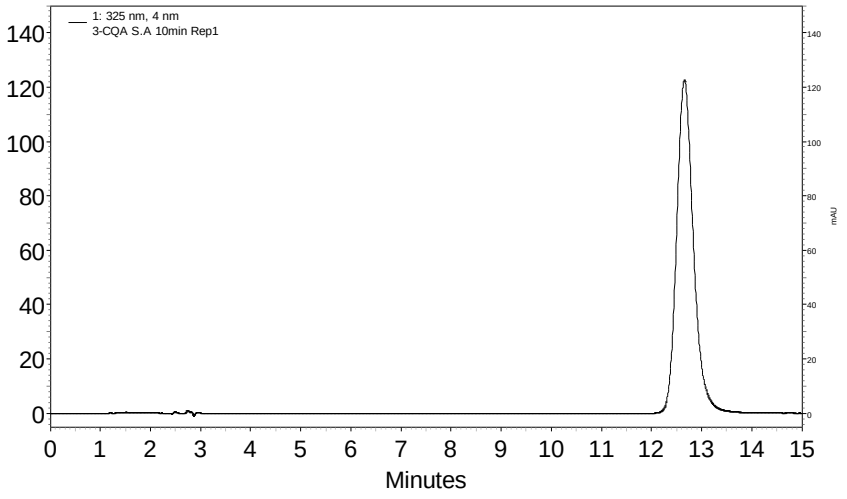


Figure 9 - Chromatogram of chlorogenic acid analyzed by HPLC-DAD at 325 nm.

The corresponding spectrum shows a maximum absorption of chlorogenic acid at 325 nm proving none elution with the wall material of the analyzed microparticles (sodium alginate) (Figure 10).

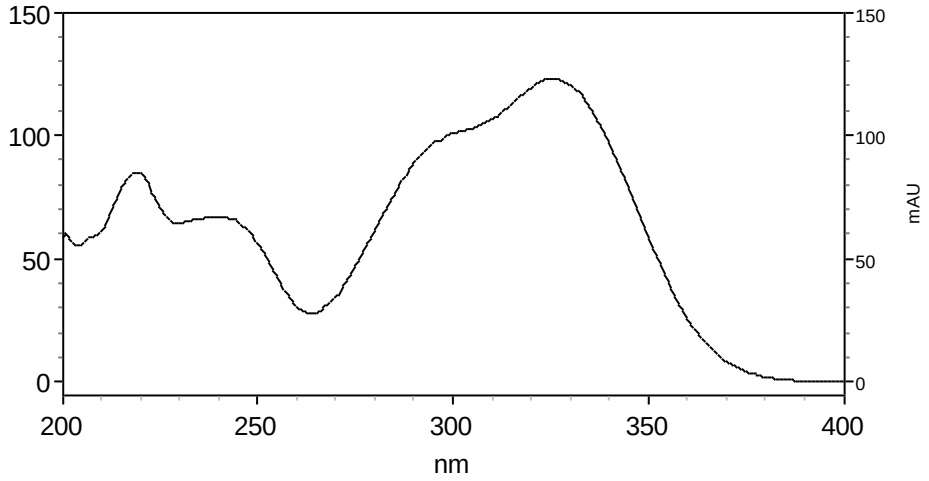


Figure 10 - Spectrum of chlorogenic acid (3-CQA) in HPLC-DAD analysis.

Regarding the detector response, the regression line was linear over the mentioned concentration range and the corresponding coefficient of correlation (R^2) of 0.999 was obtained for the analyzed compound. The sensitivity of the method is expressed as the slope of the calibration curve (Table 6 and Appendix 1 (Figure 1A)).

The limit of detection (LOD) and limit of quantification (LOQ) were calculated at signal to noise ratio of three (S/N=3) and ten (S/N=10) through the following equation (Equation 1):

$$LOD, LOQ = C \times \frac{S}{N} \times \frac{N}{H} \qquad \textbf{(Equation 1)}$$

S/N is the signal/noise ratio, C is the sample concentration, N the noise value when the blank is analysed, and H the value of the signal when the sample is analysed. The obtained values for LOQ and LOD were clearly low once both values were less than the lowest concentration of the calibration curve (Table 6). Table 6 presents the quantification parameters of the validated method.

Table 6 - Quantification parameters of HPLC-DAD method.

LOD ($mg.L^{-1}$)	LOQ ($mg.L^{-1}$)	Sensitivity ($L.mAU.mg^{-1}$)	Linearity (R^2)
0.192	0.641	117824	0.999

Since for validation of the results of a calibration curve it is required:

- at least 5 different standard concentrations
- a linearity range in a factor superior to 10
- $R^2 \geq 0.999$
- $\frac{s_a}{a} \leq 5\%$
- $\mathrm{b} - ts_b < 0 < \mathrm{b} + ts_b$

with R^2 as the correlation coefficient, a as the sensivity, b as the intercept of the regression and s_a and s_b as the respective standard deviations, the obtained results presented to be satisfactory once the linearity requirements were all accomplished (Table 7).

Table 7 – Linearity conditions for the validation of the HPLC-DAD calibration curve.

Linearity conditions
Standard concentrations: 12
Linearity factor range : 400
Correlation coefficient: 0.9999
Slope error ($\frac{s_a}{a} \leq 5\%$): 0.19%
Intercept confidence interval: - 57121 < 0 < 15777

Precision was estimated by testing the repeatability (intra-day precision) and reproducibility (inter-day precision) of 3-CQA standard solutions. For that, three concentration levels of the standard solutions (10 $mg.L^{-1}$, 75 $mg.L^{-1}$ and 150 $mg.L^{-1}$) were injected six times on the same day and three times on three consecutive days, respectively.

Precision can be expressed as relative standard deviation (RSD) or coefficient of variation (CV %), and these values should be less than 5%. From the obtained results it can be concluded that the precision was satisfactory, once all intra-day and inter-day precision results showed coefficients of variation values lower than 5%. In general, there is an increase in precision with increasing concentrations and, once for higher concentrations were obtained lower CV (%) values, the results proved to be good. Furthermore, the detector showed no significant response variations. Results of the intra-day and inter-day precisions are presented in Table 8 and in details in Appendix 2 (Table 2A and Table 2D).

Table 8 – Repeatability and reproducibility (intra-day and inter-day precision) of HPLC-DAD method.

	Intra-day precision (CV%)			Inter-day precision (CV%)		
Analyte	10 mg.L^{-1}	75 mg.L^{-1}	150 mg.L^{-1}	10 mg.L^{-1}	75 mg.L^{-1}	150 mg.L^{-1}
Chlrogenic acid (3-CQA)	1.45	1.06	0.46	1.31	0.51	0.32

4.1.2. UV-Vis Spectrophotometry

Regarding UV-vis spectrophotometry, the absorption spectrum of chlorogenic acid was determined for both controlled release mediums (pH 5.6 and pH 2) in order to obtain the wavelength of maximum absorption of 3-CQA. Figure 8 presents the obtained spectra for both pH values. The maximum absorption of 323 nm was observed for water at pH 5.6 (Figure 11a). For water at pH 2, the maximum absorption was obtained at 324 nm (Figure 11b). Considering both results, detection was performed by measuring the absorption at these wavelengths in the respective spectrophotometry analyses.

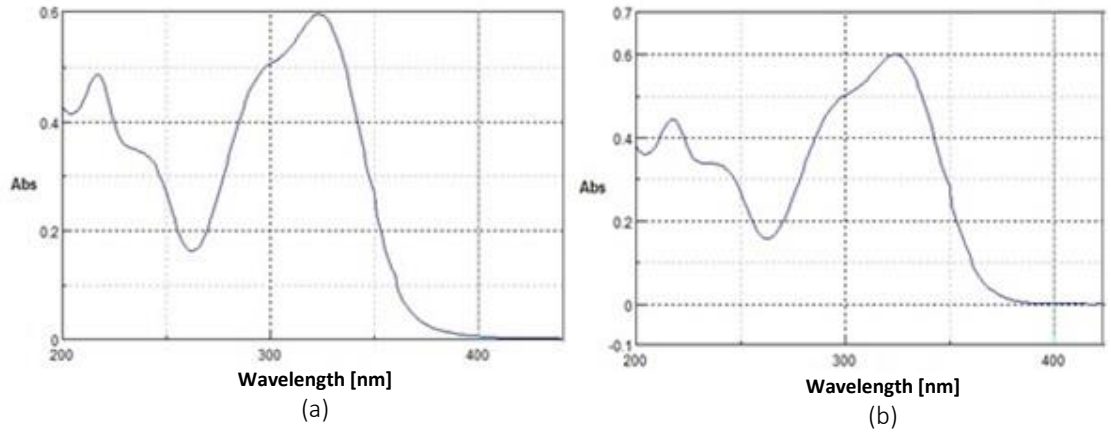


Figure 11 - Absorption spectrum of chlorogenic acid (3-CQA) for water at: (a) pH 5.6; (b) pH 2.

Two calibration curves were prepared: one for desionised water at normal pH (pH 5.6) and another for aqueous solution at pH 2.0. For that, the absorption values (AU) were plotted against the corresponding concentrations (mg.L^{-1}) by duplicated measurement of 1 mL of the chlorogenic acid standard solutions at seven concentration levels between 0.5 and 15 mg.L^{-1} (0.5, 1, 2, 5, 8, 10 and 15 mg.L^{-1}). The regression line obtained through the detector response was linear over the mentioned concentration range and the corresponding coefficients of correlation (R^2) were 0.999. The sensitivity of the method is expressed as the slope of the calibration curves and presented to be the same in both cases (Table 9 and Appendix 1 (Figures

1B and 1C)). Comparing to HPLC-DAD method, UV-Vis spectrophotometry proved to be more sensitive for chlorogenic acid (3-CQA) analysis.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the response and on the slope of the calibration curve, through the following expressions (Equation 2 and 3):

$$LOD = \frac{3 \times s_b}{a} \quad \text{(Equation 2)}$$

$$LOQ = \frac{10 \times s_b}{a} \quad \text{(Equation 3)}$$

The obtained values for LOQ and LOD were satisfactory low, however in case of water at pH 5.6 results were not so good once they were lightly higher than the lowest concentration of the calibration curve (Table 9).

Table 9 - Quantification parameters of UV-vis spectrophotometry.

Analyte	Sensitivity (L.AU.mg^{-1})	Linearity (R^2)	LOD (mg.L^{-1})	LOQ (mg.L^{-1})
Chlorogenic acid (3-CQA) in water pH 5.6	0.072	0.999	0.18	0.60
Chlorogenic acid (3-CQA) in water pH 2	0.074	0.999	0.09	0.31

Precision was assessed by testing the repeatability (intra-day precision) of three different standard solutions (2 mg.L^{-1}, 8 mg.L^{-1} and 10 mg.L^{-1}) six times in the same day and by intermediate precision (inter-day precision), analyzing the same three standard solutions three times on three consecutive days. Results of the intra-day and inter-day precisions are presented in Table 10 and in details in Appendix 2.

Table 10 - Repeatability and reproducibility (intra-day and inter-day precision) of UV-Vis spectrophotometry.

	Intra-day precision (CV%)			Inter-day precision (CV%)		
Analyte	2 mg.L^{-1}	8 mg.L^{-1}	10 mg.L^{-1}	2 mg.L^{-1}	8 mg.L^{-1}	10 mg.L^{-1}
Chlrogenic acid (3-CQA) in water pH 5.6	4.75	1.06	0.39	0.04	0.01	0.01
Chlrogenic acid (3-CQA) in water pH 2	0.28	1.06	0.15	0.01	0.01	0.01

From the obtained results it can be concluded that the precision was satisfactory, once all intra-day and inter-day precision results showed coefficients of variation values lower than 5%.

However there were some response variations between analysis in water at pH 5.6 and water a pH 2, mainly for the lowest concentration in intra-day and inter-day precisions.

4.2. Characterization of chlorogenic acid microparticles

4.2.1 Product yield (%)

Sodium alginate and modified chitosan were both used for microencapsulation of chlorogenic acid (3-CQA) by spray drying process. The product yield obtained (quantity of powder recovered reported to the quantity of raw materials) was 41.09% for sodium alginate and 39.29% for modified chitosan which are satisfactory results regarding the technique applied and the scale used. Once that the amount of raw materials used was small, comparing with the equipment size, losses were obtained. On the other hand, the size of the obtained microparticles is very small allowing their suction by the vacuum system. Similar results were obtained by Estevinho et al., 2014 for microencapsulation of β-galactosidase by spray drying into different biopolymeric materials. Values ranged from 36% to 59% depending on the biopolymer used. The product yield for preparation of microparticles using sodium alginate and modified chitosan was 36% and 59%, respectively.

4.2.2 Particle size distribution

Results obtained for size distribution in number (%) and in volume (%) of chlorogenic acid (3-CQA) microparticles produced with sodium alginate and modified chitosan are presented in Figure 12a and Figure 12b, respectively. Through the graphs is possible observe that, for both type of samples, the average value (from duplicates) of the particle size obtained for volume distribution was around 3 μm (2.8 μm and 3.2 μm for sodium alginate a modified chitosan microparticles, respectively). For size distribution in number, was verified a particle size less than 1 μm again for both kinds of the prepared microparticles. In case of sodium alginate the particles mean size was 0.7 μm, for modified chitosan was 0.9 μm.

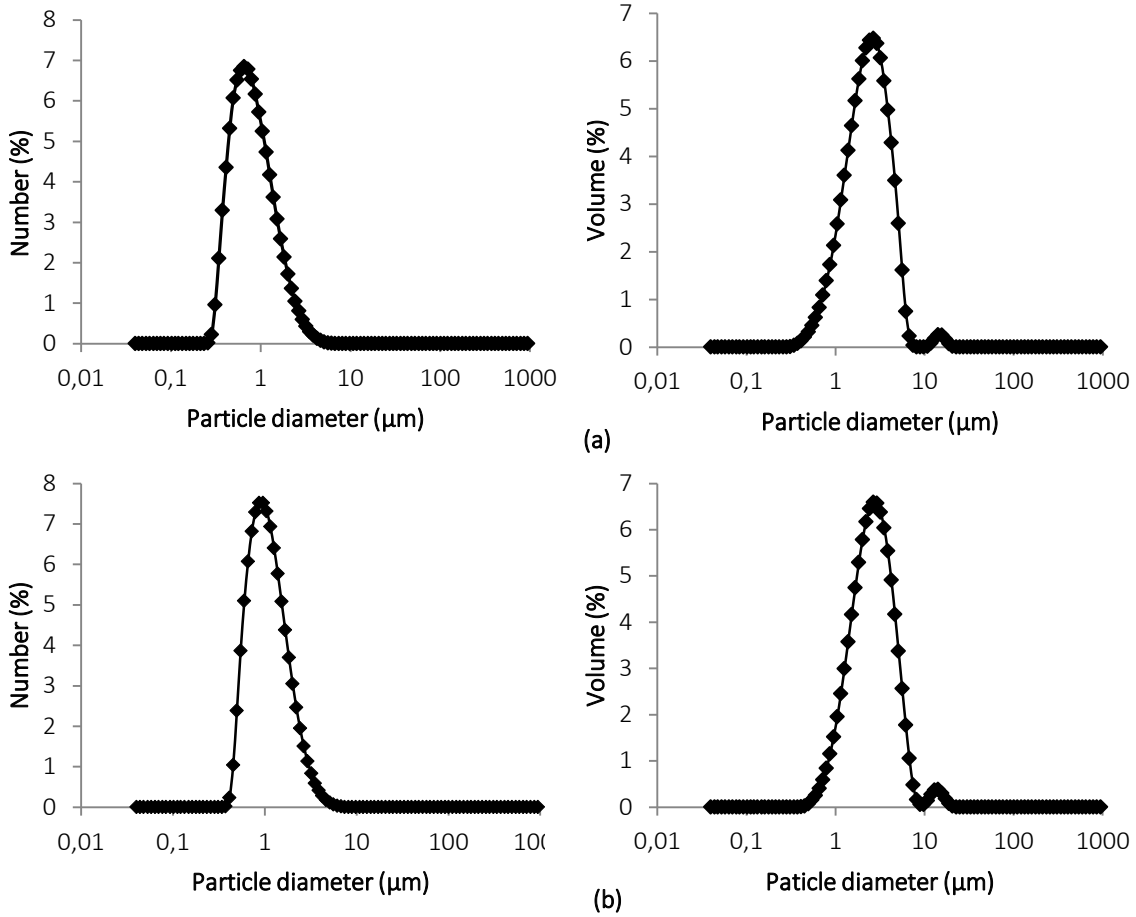


Figure 12: Particle size distribution in number (%) and in volume (%) of chlorogenic acid (3-CQA) microparticles produced with: (a) sodium alginate; (b) modified chitosan.

Regarding the obtained results it is possible to conclude that, in the present study, the particles size of the prepared microparticles was not affected by the kind of wall material used since that similar mean diameter was obtained. In addition, the results obtained for particles size distribution in number revealed that the obtained microparticles are mainly small. Since number and volume distribution are close, distribution presented to be homogeneous. Although a small amount of microparticles present a large size, which may be explained by aggregates formation.

4.2.3 Scanning Electron Microscopy (SEM) analysis

The chlorogenic acid loaded microparticles were analyzed by scanning electron microscopy for analysis of particle morphology. SEM images of the microparticles prepared with sodium alginate (A_1 and A_2) and with modified chitosan (B_1 and B_2) are shown in Figure 13. As it is

possible to observe, spherical microparticles with a smooth surface were produced in both cases. However, in the case of the sodium alginate microparticles their shape presented to be not so regular than the microparticles prepared with modified chitosan.

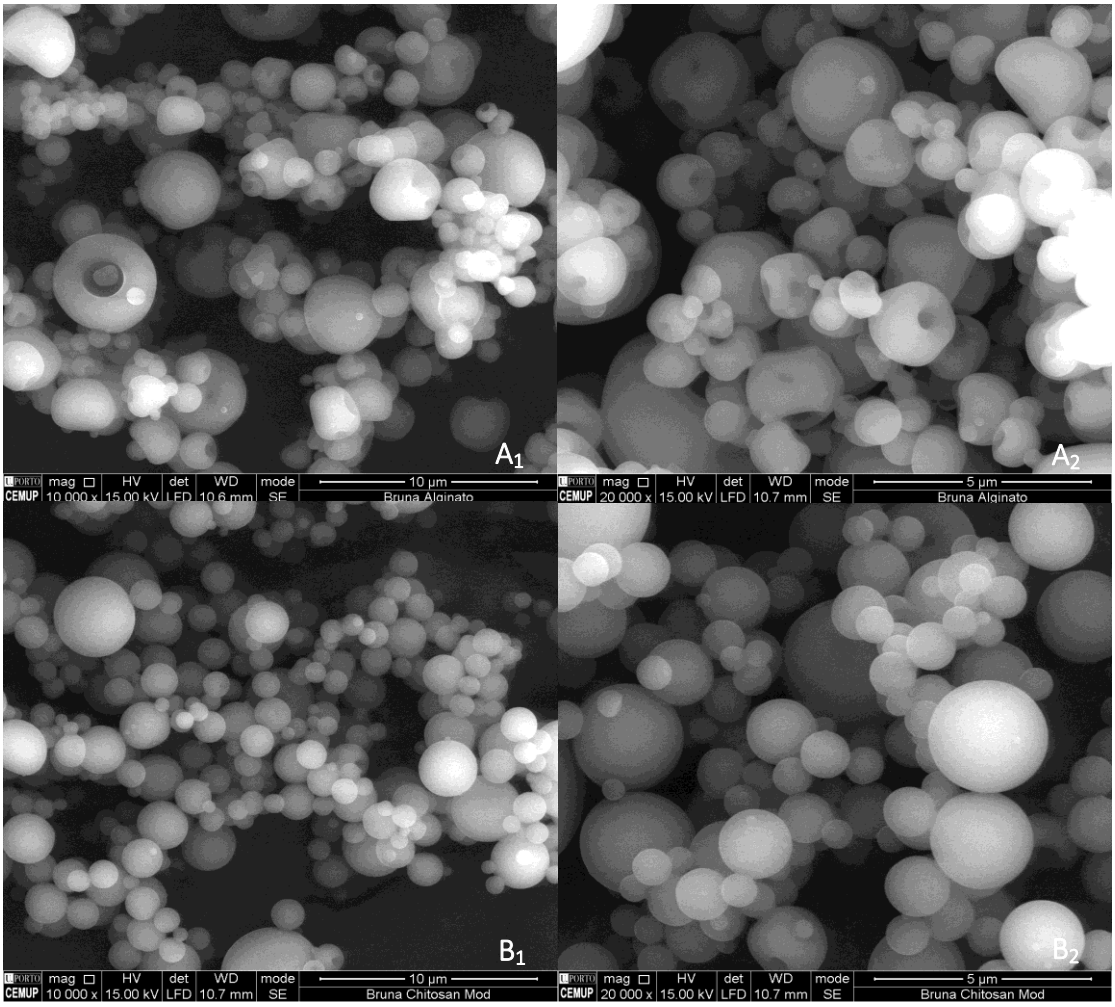


Figure 13: SEM images of the microparticles prepared with (A) sodium alginate and (B) modified chitosan. Amplification of (A_1,B_1) 10 000 and (A_2,B_2) 20 000 times, beam intensity (HV) of 1500 kV, distance between the sample and the lens (WD) around 10 mm.

Other authors have reported the same morphology for spray dried microparticles of sodium alginate and modified chitosan (Estevinho et al., 2014).

4.3 Controlled release studies of chlorogenic acid (3-CQA)

The controlled release study of chlorogenic acid (3-CQA) from the obtained microparticles was evaluated at two different pH values (pH 5.6 and pH 2). Thus, the release profile of sodium alginate and modified chitosan microparticles was individually determined in aqueous solution at pH 5.6 and pH 2 at room temperature, over 24h. Two methods, HPLC-DAD and UV-Vis

spectrophotometry, were used for quantification of the loaded chlorogenic acid after each defined time interval from the referred microparticles under the referred conditions.

4.3.1 HPLC-DAD

The instrumental analysis for chlorogenic acid quantification was firstly performed by the HPLC-DAD method validated previously. Through this method it was possible to determine the release profile of chlorogenic acid from sodium alginate microparticles in water at pH 5.6. Figure 14 illustrates the obtained release profile in terms of percentage release (%) of chlorogenic acid under the referred conditions.

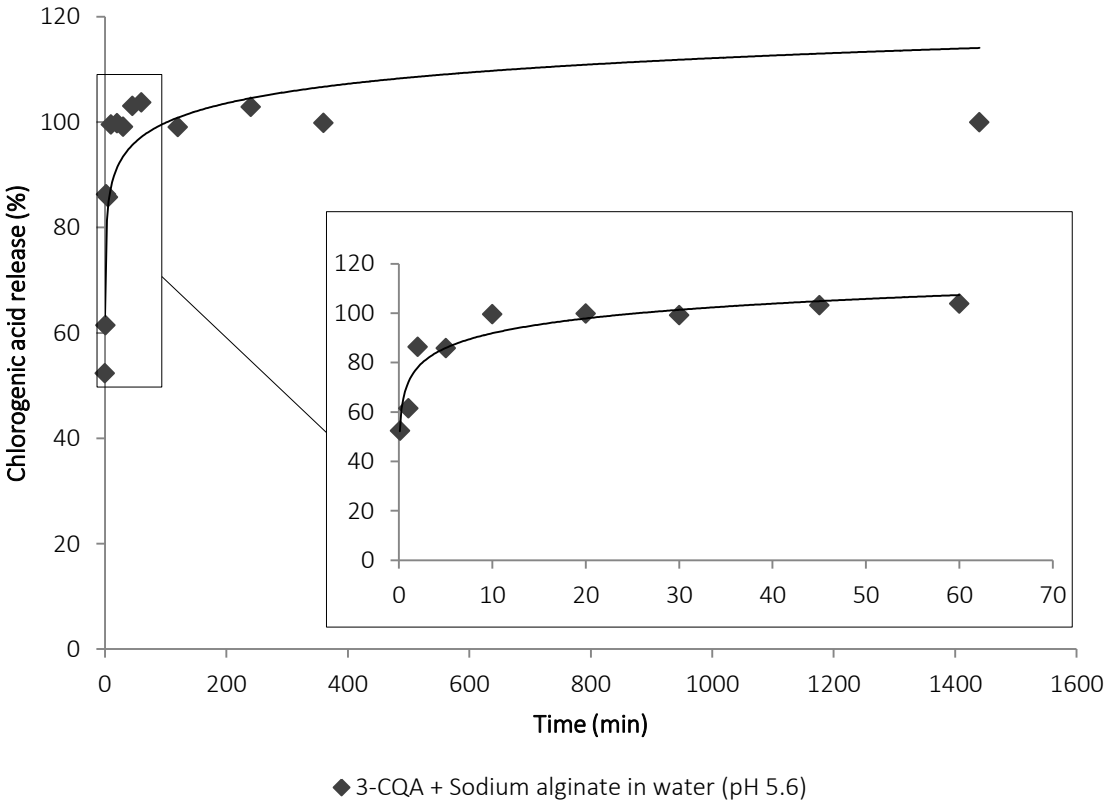


Figure 14 - Release profile of 3-CQA (%) from sodium alginate microparticles in water at pH 5.6.

As it is possible to observe, sodium alginate (SA) microparticles presented a fast release pattern (99%) during the first 10 min, followed by a continuous release till 24h. This result is consistent with what has been reported in the literature once that was referred that sodium alginate is easily disintegrated under higher pH conditions (Yoo et al., 2006).

The same experiment was planned for chlorogenic acid release at pH 2. However due to some properties of sodium alginate, such as viscosity, the stability of the chromatographic

column was compromised. Microparticles caused HPLC blocking and damage and, because of that, it was impossible to proceed with the desired experiment using HPLC-DAD as analytical method. For that, a UV-Vis spectrophotometry method was developed, validated and used for chlorogenic acid determination.

4.3.2 UV-Vis Spectrophotometry

UV-Vis spectrophotometry was used to determine the release profile of chlorogenic acid (3-CQA) from sodium alginate and modified chitosan microcapsules at two different pH values, exactly at same conditions mentioned previously.

Figure 15 presents the release profile obtained by UV-Vis spectrophotometry in terms of percentage release (%) of chlorogenic acid (3-CQA) at pH 5.6 and pH 2.0 using sodium alginate as microencapsulation wall material.

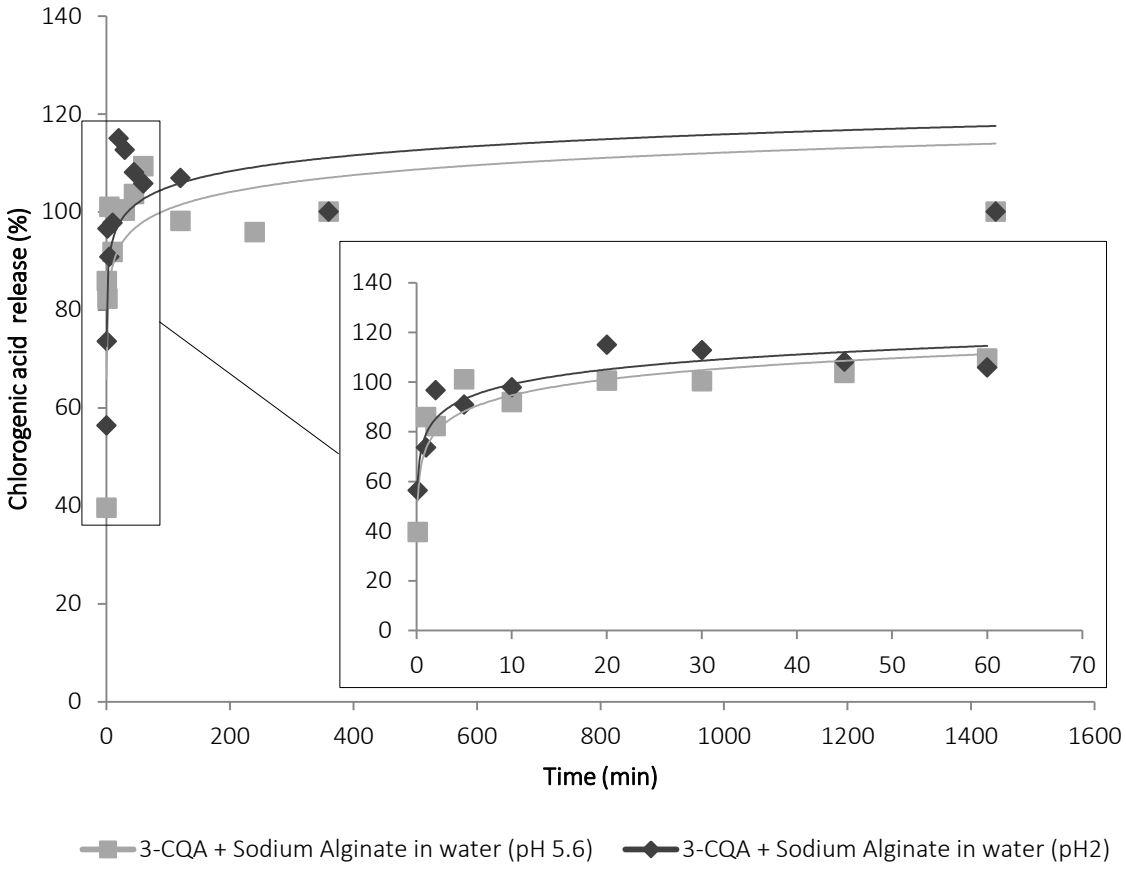


Figure 15 - Release profile of 3-CQA (%) from sodium alginate microparticles in water at two different pH values (pH 5.6 and pH 2).

Through the graph is possible to see that, as in HPLC-DAD results, SA microparticles also presented a fast release pattern (90%), during the first 10 min, being followed by a continuous release till 24h. The same pattern was verified for both pH values proving that the release rate of chlorogenic acid from the SA-based microparticles was not significantly affected by pH.

Regarding Yoo et al., 2006 study, sodium alginate was used as coating material for microencapsulation of an antioxidant of a vitamin E category (α-tocopherol) by ionic gelation and its *in vitro* releasing property proved to be relatively different. In the simulated gastric fluid (0.05M HCl with 0.2% NaCl) the SA microcapsules release was less (28.8%) than when exposed to a simulated intestinal fluid (0.05M phosphate buffer) where the amount of the released α-TP was about 81.5%. The same behavior was reported by Kumar and Krishna, 2014 when sodium alginate was used for encapsulation of an anti-diabetic drug (Vildagliptine) for sustained release in three different dissolution mediums (pH 1.2, pH 6.8 and pH 7.4). In their study, the prepared microspheres showed an increased drug release at higher pH (pH 7.4 and pH 6.8) than in lower pH values (pH 1.2). Although sodium alginate has been reported as relatively stable at acidic pH (pH 1.2) but easily disintegrated under mild alkali conditions (pH 7.4 and pH 6.8), in the present study results revealed none interference of pH on the release rate of chlorogenic acid (3-CQA) from SA-based microparticles.

Regarding the release profile of 3-CQA from microcapsules performed with modified chitosan at two different pH values (pH 2 and pH 5.6), similar results were obtained (Figure 16).

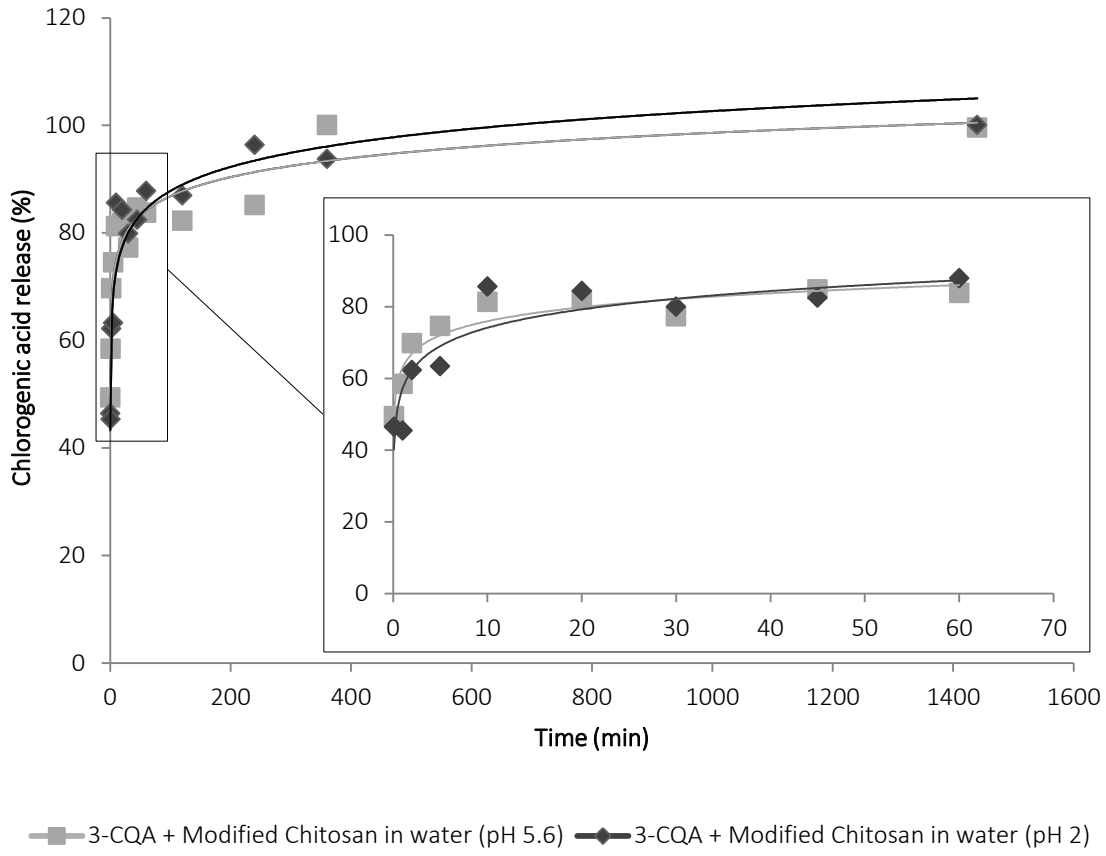


Figure 16 - Release profile of 3-CQA (%) from modified chitosan microparticles in water at two different pH values (pH 5.6 and pH 2).

Figure 16 shows that, as sodium alginate, also modified chitosan microcapsules presented a fast release pattern (80%) during the same time range (10 min), followed by a continuous release till 24h. In this case, the complete release of 3-CQA was achieved after 4h and the same pattern was verified for both pH values. As for SA microparticles, the obtained release rate of chlorogenic acid from the modified chitosan microparticles was not significantly affected by pH. However this behavior was not expected once that, according to George, 2006, the main goal of modyfing chitosan chemically is to provide derivatives with higher solubility at neutral and basic pH values. Nallamuthu et al., 2014 reported the release profile of chlorogenic acid from microparticles prepared with chitosan and the same behavior was obtained: the release of chlorogenic acid was faster in neutral pH (PBS, pH 7.4) than lower pH values (HCl, pH 2). On the other hand, Shi et al., 2007 revealed the release pattern of chlorogenic acid from yeast-encapsulated cells and it was verified a behavior significantly different where the release rate of chlorogenic acid in simulated gastric fluid (HCl, pH 1.2) was higher than in distilled water and in intestine conditions (PBS, pH 7.4). The difference between these results confirms the significant influence of the wall material in the release pattern of the encapsulated compounds, in this particular case, for chlorogenic acid controlled release.

Conclusions

In this study, sodium alginate and modified chitosan-loaded microparticles of chlorogenic acid (3-CQA) were prepared by spray drying in order to enhance its stability and bioavailability for possible food applications. The obtained microparticles were characterized by their particle size and surface morphology and their controlled release profile was studied in aqueous solution at two different pH values (pH 5.6 and pH 2). Methods for analytical detection and quantification (HPLC-DAD and UV-Vis spectrophotometry) of chlorogenic acid were developed and validated.

Results proved that chlorogenic acid was successfully encapsulated into sodium alginate and modified chitosan using spray drying technique and a satisfactory product yield was obtained for encapsulation in both wall materials (41.09% and 39.29% for sodium alginate and modified chitosan, respectively).

All the parameters from validation of the analytical methods (HPLC-DAD and UV-spectrophotometry) showed that both methods were sensitive and precise with low detection and quantification limits. However, UV-Vis spectrophotometry proved to be more sensitive than HPLC-DAD for detection and quantification of chlorogenic acid (3-CQA). Both methods were used to investigate the release profile of chlorogenic acid from both types of microparticles: sodium alginate and modified chitosan-loaded microparticles.

Controlled release profiles evaluated through UV-Vis spectrophotometry revealed a fast release of chlorogenic acid (80%-90% after 10 min) from both wall materials at both pH values. Although, comparing to sodium alginate microparticles, microparticles produced with modified chitosan presented a slower release profile. Furthermore, it was also possible to see that the release rate of chlorogenic acid (3-CQA) was not significantly affected by pH changes.

Both types of produced microparticles were small in size (3 μm) and presented a spherical shape and smooth surface. However modified chitosan showed a more regular shape.

Collectively, these results suggest that microparticles synthesized by spray drying using sodium alginate and modified chitosan as encapsulating agents can be used in food-matrices for health benefits through effective release of chlorogenic acid.

Limitations and Future Work

Some limitations were faced during the development of this work. Availability of HPLC and UV-spectrophotometer was one of the majors since these equipments were also been used by other researchers in their projects. Furthermore, analysis through HPLC presented to be really time consuming regarding the necessity to inject samples one by one (in duplicated) after the defined time intervals for the controlled release studies together with a washing run of at least 20 min to clean the column between each single injection. Besides that, HPLC column blocking by microparticles obliged to the development and validation of a new method for chlorogenic acid quantitative determination, delaying all the results.

For future work it would be interesting attempt for a suitable extraction method for phenolic compounds (including chlorogenic acids) from the most prominent species of coffee (*Coffea arabica* and *Coffea robusta*) for further encapsulation. The obtained microparticles should be characterized and analyzed in order to achieve their release profile and evaluate their antioxidant activity. *In vitro* studies could also provide relevant information about the therapeutic effects of chlorogenic acid microparticles regarding the antimicrobial activity related to this phenolic compound. Furthermore, others encapsulating agents should be tested for encapsulation of chlorogenic acids. More controlled release studies should be done, particularly, in commercial food-matrices for real data acquisition.

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Appendix 1 – Calibration curves

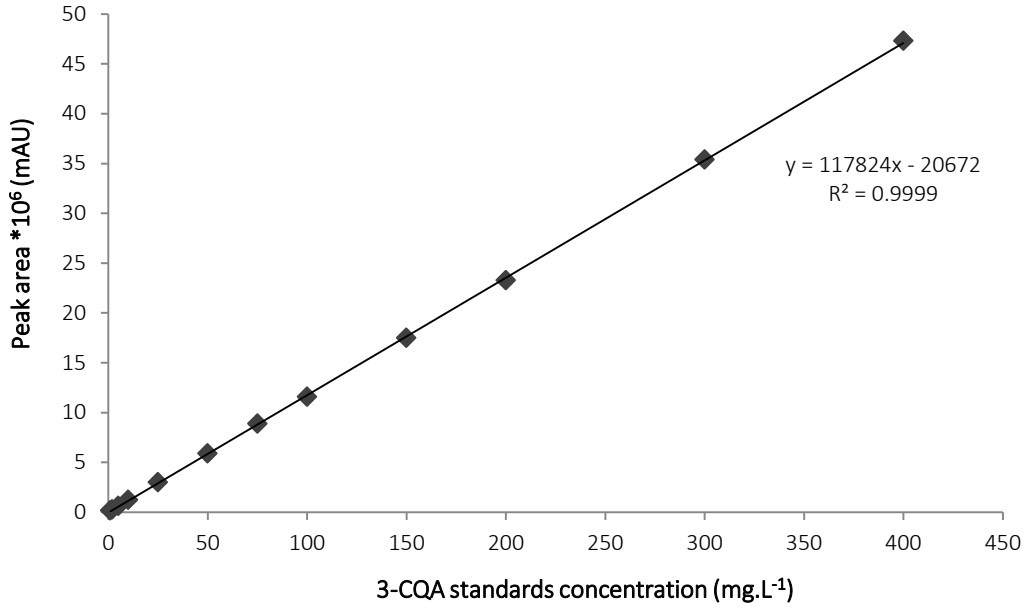

Figure 1A - Calibration curve of chlorogenic acid (3-CQA) in linearity rage of 1 - 400 mg.L^{-1} (HPLC-DAD; pH 5.6).

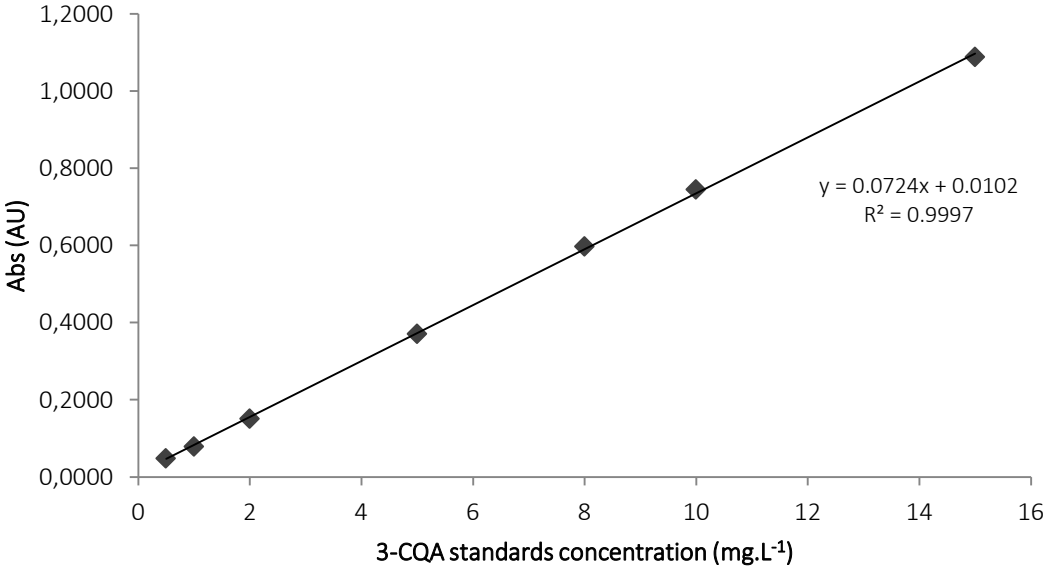

Figure 1B - Calibration curve of chlorogenic acid (3-CQA) in linearity rage of 0.5 - 15 mg.L^{-1} (UV-vis spectrophotometry; pH 5.6).

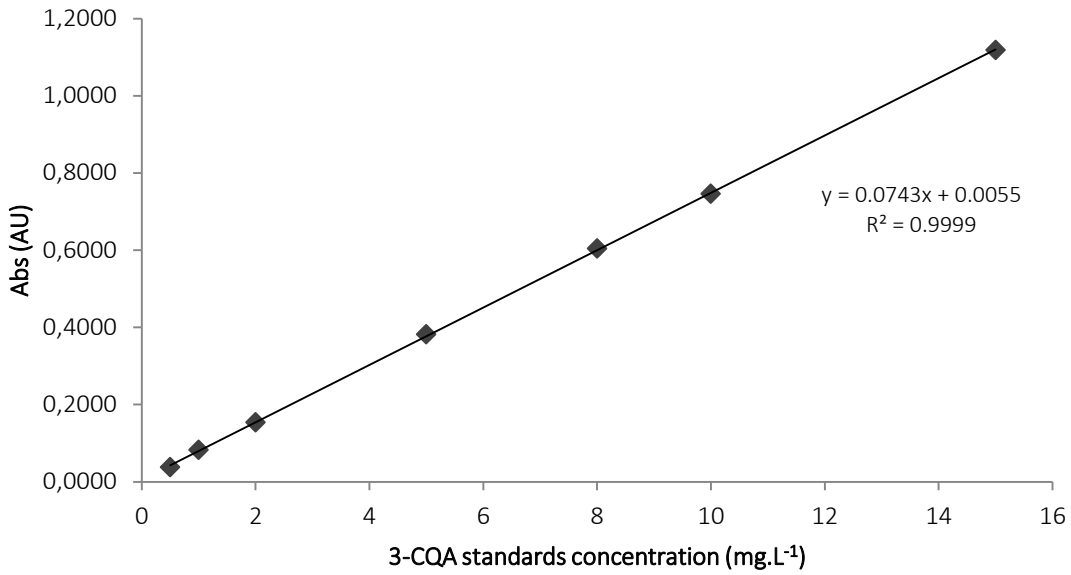

Figure 1C - Calibration curve of chlorogenic acid (3-CQA) in linearity rage of 0.5 - 15 $mg.L^{-1}$ (UV-vis spectrophotometry; pH 2).

Appendix 2 - Precision

Table 2A - Intra-day precision (%CV) of the HPLC-DAD method for chlorogenic acid (3-CQA), evaluated from calibration curve (pH 5.6).

3-CQA ($mg.L^{-1}$)	Repetition (n)	Peak area (mAU)	Average	CV%
10	1	1243742	1210191.67	1.45
	2	1201038		
	3	1204352		
	4	1199189		
	5	1198085		
	6	1214744		
75	1	8848633	8809013.50	1.06
	2	8892006		
	3	8762177		
	4	8620646		
	5	8928364		
	6	8802255		
150	1	17470212	17447357.67	0.46
	2	17479474		
	3	17461528		
	4	17560496		
	5	17378593		
	6	17333843		

Table 2B - Intra-day precision (%CV) of the UV-vis method for chlorogenic acid (3-CQA), evaluated from calibration curve (pH 2).

3-CQA ($mg.L^{-1}$)	Repetition (n)	Abs (AU)	Average	CV%
2	1	0.1530	0.15	0.28
	2	0.1534		
	3	0.1535		
	4	0.1537		
	5	0.1543		
	6	0.1538		
8	1	0.6031	0.60	1.06
	2	0.6038		
	3	0.6022		
	4	0.6026		
	5	0.6045		
	6	0.6050		
10	1	0.7440	0.75	0.15
	2	0.7455		
	3	0.7447		
	4	0.7455		
	5	0.7467		
	6	0.7469		

Table 2C - Intra-day precision (%CV) of the UV-vis method for chlorogenic acid (3-CQA), evaluated from calibration curve (pH 5.6).

3-CQA ($mg.L^{-1}$)	Repetition (n)	Abs (AU)	Average	CV%
2	1	0.1651	0.15	4.75
	2	0.1502		
	3	0.1485		
	4	0.1490		
	5	0.1464		
	6	0.1459		
8	1	0.5959	0.60	1.60
	2	0.5943		
	3	0.5938		
	4	0.6036		
	5	0.5989		
	6	0.5953		
10	1	0.7467	0.74	0.39
	2	0.7428		
	3	0.7455		
	4	0.7402		
	5	0.7484		
	6	0.7452		

Table 2D - Inter-day precision (%CV) of the HPLC-DAD method for chlorogenic acid (3-CQA), evaluated from calibration curve.

Sample	Day	Repetition (n)	C1 (10 mg.mL^{-1})	CV%	Average CV%	C2 (75 mg.mL^{-1})	CV%	Average CV%	C3 (150 mg.mL^{-1})	CV%	Average CV%
3-CQA	1	1	1243742	1.95	1.31	8848633	0.75	0.51	17470212	0.05	0.32
		2	1201038			8892006			17479474		
		3	1204352			8762177			17461528		
	2	1	1231603	0.56		9047862	0.29		17570747	0.62	
		2	1224790			9002045			17626628		
		3	1238702			9004421			17781055		
	3	1	1222904	1.42		8824601	0.50		17571766	0.29	
		2	1188999			8743187			17545988		
		3	1202775			8754791			17644293		

Table 2E - Inter-day precision (%CV) of the UV-vis method for chlorogenic acid (3-CQA), evaluated from calibration curve (pH 2).

Sample	Day	Repetition	C1 (2 mg.mL^{-1})	CV%	Average CV%	C2 (8 mg.mL^{-1})	CV%	Average CV%	C3 (10 mg.mL^{-1})	CV%	Average CV%
3-CQA	1	1	0.1455	0.010	0.007	0.5797	0.004	0.006	0.7289	0.004	0.003
		2	0.1483			0.5751			0.7240		
		3	0.1475			0.5763			0.7284		
	2	1	0.1432	0.003		0.5544	0.002		0.6931	0.001	
		2	0.1439			0.5564			0.6936		
		3	0.1434			0.5544			0.6927		
	3	1	0.1425	0.009		0.5450	0.010		0.6770	0.004	
		2	0.1417			0.5342			0.6821		
		3	0.1441			0.5420			0.6809		

Table 2F - Inter-day precision (%CV) of the UV-vis method for chlorogenic acid (3-CQA), evaluated from calibration curve (pH 5.6).

Sample	Day	Repetition	C1 (2 mg.mL^{-1})	CV%	Average CV%	C2 (8 mg.mL^{-1})	CV%	Average CV%	C3 (10 mg.mL^{-1})	CV%	Average CV%
3-CQA	1	1	0.1466	0.041	0.038	0.5874	0.003	0.004	0.7368	0.004	0.007
		2	0.1360			0.5869			0.7371		
		3	0.1457			0.5897			0.7321		
	2	1	0.1945	0.030		0.6227	0.005		0.7713	0.009	
		2	0.1860			0.6278			0.7685		
		3	0.1836			0.6289			0.7812		
	3	1	0.1109	0.044		0.4445	0.003		0.5621	0.007	
		2	0.1210			0.4453			0.5549		
		3	0.1165			0.4471			0.5615		